ISOLATION AND CLONING OF GENES ASSOCIATED WITH APOMIXIS IN *CENCHRUS CILIARIS*

THESIS

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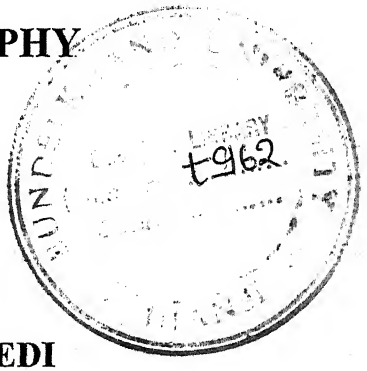

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CERTIFICATE

It is certified that the thesis entitled " Isolation and cloning of genes associated with apomixis in *Cenchrus ciliaris*" is an original piece of work done by Mr. Krishna Kumar Dwivedi, M.Sc. (Biotechnology) under my supervision and guidance for the degree of Doctor of Philosophy in Biotechnology, Bundelkhand University, Jhansi.

I, further certify that:

- It embodies the original work of the candidate himself.
- It is up to the required standard both in respect of its contents and literary presentation for being referred to the examiners.
- The candidate has worked under me for the required period at Indian Grassland and Fodder Research Institute, Jhansi.
- The candidate has registered the required attendance at Indian Grassland and Fodder Research Institute, Jhansi.
- Besides isolation and cloning of genes, this study also contains studies on protein and isozyme polymorphism.

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DECLARATION

I, hereby, declare that the thesis entitled " Isolation and cloning of genes associated with apomixis in *Cenchrus ciliaris*" submitted by me for the award of degree of Doctor of Philosophy in Biotechnology, Bundelkhand University, Jhansi, is the original piece of work done by me under the supervision of Dr. Vishnu Bhat, Scientist (Sr.Scale), I.G.F.R.I. Jhansi and to the best of my knowledge either a part or whole of this thesis work has not been submitted for any degree or any other qualification of any university or examining body in India or any university elsewhere.

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CHAPTER 1
INTRODUCTION

Apomixis in flowering plants is defined as the asexual formation of seed from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization, leading to embryo development (Bicknell *et al,* 2004). Winkler (1908) introduced the term apomixis to mean "substitution of sexual reproduction by an asexual multiplication process without nucleus and cell fusion". In apomictic plants, meiosis is circumvented during the differentiation of the female gametophyte from megaspore mother cell, or meiotic derivatives degenerate (Nogler, 1984a; Asker and Jerling, 1992). Apomixis provides a method for cloning plants through the seed. The advantages of apomixis are: (1) the fixation of any genotypic combination; (2) propagation of hybrids through seed progeny (permanent hybrid); (3) the maintenance of unique chromosome aberrations and (4) the production of true-to-type seeds. The most effective use of apomixis is for the commercial production of hybrid cultivars that exhibits hybrid vigour. The reuse of hybrid seeds are extremely valuable because heterosis can be perpetuated through successive generation via seed. In other words, prized hybrid seeds once produced can be multiplied "vegetatively" via apomixis at no additional cost to the farmers". It is this attractive prospect that has awakened worldwide interest in introducing apomixis into crop plants.

Apomixis is widely distributed amongst higher plants. More than 400 species belonging to 40 families are apomictic. It is most common in *Gramineae, Compositae, Rosaceae, Rutaceae* (Carman, 1997). As a rule, with a few exceptions, apomixis is more prevalent in polyploids. It is widespread in several forage grass genera such as *Cenchrus, Paspalum, Poa, Bothrichloa* and *Dichanthium* and most of them are polyploids. Buffel grass (*Cenchrus ciliaris*) is a tropical, perennial forage grass species with extensive range from Africa to India (Bogdan, 1977). It is an inhabitant of drier sandy area throughout Africa, Canary Island, Madagascar and eastward to India. It is well adapted to semi-arid conditions, has better persistence and offers resistance to trampling and withstands frequent cutting. In contrast to other apomictic grasses, most buffel grass genotypes

reproduce by obligate apospory and aposporous unreduced (2n) female gametophyte can be cytologically identified, because they lack antipodal cells (Young and Bashaw, 1979). *Cenchrus ciliaris* reproduce by apospory associated with pseudogamy (Bashaw, 1962). Although the numbers of chromosomes have been reported to range from 32 to 54 (Fisher *et al.*, 1954), most genotypes are tetraploids (2n=4x=36). Cytological observations have indicated that the 36-chromosome types are segmental allotetraploids with 10–14 bivalents and 2–4 quadrivalents at diakinesis (Fisher *et al.*, 1954; Snyder *et al.*, 1955). Segregation studies of apomixis in buffelgrass have reported both disomic (Taliaferro and Bashaw, 1966) and tetrasomic (Sherwood *et al.*, 1994) inheritances, with potential for recessive lethality. Bashaw (1962) reported the discovery of first sexual plant in *C. ciliaris*. The aposporous embryo sacs contained three celled egg apparatus and a single polar nucleus, while antipodals were absent. Such embryo sacs (four nucleate sacs) are always unreduced. The sexual embryo sacs are always reduced, and are always eight nucleate and bipolar. Based on this principle, Gupta *et al.* (2001) identified two species of *Cenchrus* and a plant of *C. ciliaris* as sexually reproducing, after screening a global germplasm.

Apomictic processes mimic many of the events of sexual reproduction and give rise to fertile seeds. An important difference is that the apomictic embryo is derived solely from cells in the maternal ovule tissues rather than from fusion of male and female gametes. The seeds that result from apomictic reproduction contain embryos that have barring mutation, a genetic constitution identical to that of the female parent. At least three developmental differences also serve to distinguish apomictic embryo formation from somatic embryogenesis. First, apomictic embryo formation occurs within a differentiated structure. Second, apomictic embryos form directly from a cell located in, or close to, a gametophytic structure, without entering an intervening callus phase, which is often necessary for somatic embryogenesis (Nomura and Komamine, 1985, Zimmerman, 1993). Third, the pattern of embryo formation in apomictic species is often indistinguishable from that which occurs in the nearest sexual relative (Nogler, 1984a) which is not always the case for somatic embryogenesis.

The mechanisms of apomixis are better understood in terms of the events occurring during the female reproductive development involving megasporogenesis and megagametogenesis. During normal plant reproduction, a defined sequence of events in the ovule leads to the development of haploid eggs, which when fertilized by male

gametes result in sexual embryos. These steps involve: differentiation of megaspore mother cell (MMC), megasporogenesis through meiosis leading to the development of a haploid megaspore from the MMC and megagametogenesis resulting in embryo sac with egg apparatus, polar nuclei and antipodal cells starting from the megaspore. The ovule also consists of sporophytically derived tissues that nourish and protect the female gametophyte and developing embryo. Ultimately, the mature embryo, endosperm and maternal tissues of the ovule form the seed. The study of ovule development can yield valuable insight into general developmental mechanism, because many of the processes involved in ovule development have parallel in other eukaryotic organisms, including the establishment of polarity and pattern, lineage specific cell, nuclear division and programmed cell death (Drubin, 1991). A single cell, known as the megaspore mother cell or archesporial cell, in the hypodermal layer of the young ovule enlarges and undergoes meiosis to produce a linear tetrad of megaspores and this process is megasporogenesis. Because these four cells are the products of meiosis, their chromosome number is haploid or half of that of the adjacent somatic nucellar cells of the ovule. In apomixis, since meiosis is circumvented, megaspores contain the somatic chromosome number. In the normal process, the three megaspores nearest to the micropyle degenerate while remaining chalazal megaspore enlarges and undergoes three mitotic divisions to produce an eight nucleate embryo sac (female gametophyte) by megagametogenesis. The mature embryo sac consists of an egg cell, two synergid cells and two polar nuclei and three antipodal cells. Whereas in case of apomixis, in the mature embryo sacs, the synergids have disintegrated or not readily visible and the antipodal continue to divide to produce a cluster of cells in the chalazal end of the sac, or are absent.

There are two types of apomixis, *viz.*, gametophytic apomixis, where the gametophyte originates from unreduced cells, and sporophytic apomixis or adventitious embryony, where the unreduced cells form an embryo directly. Gametophytic apomixis is subdivided depending on whether the megagametophye is developing from an unreduced megaspore (diplospory) or from a sporophytic cell in the ovule (apospory) (Nogler, 1984a; Asker and Jerling, 1992). Different kinds of apomixis can co-exist within an individual plant (Spillane *et al.*, 2001b).

The genetic basis of apomixis is of great significance for any research aiming to transfer apomixis into non-apomictic plants. Broadly, three sets of genes may be

involved in reproduction *viz.*, progress of normal meiosis followed by mitosis of megaspore nucleus, progress of embryo sac development, consummation of fertilization and development of embryo. The inheritance of gametophytic apomixis has since been reported to be associated with the transfer of either single locus or a small number of loci in most of the systems studied to date. In the aposporous grasses *Pennisetum* (Sherwood *et al.*, 1994), *Panicum* (Savidan, 1983), and *Brachiaria* (Valle *et al.*, 1994), apomixis is reported to be simply inherited with the trait conferred by the transfer of a single dominant factor. Simple dominant inheritance also has been reported for apospory in the dicotyledonous genera *Ranunculus* (Nogler, 1984b) and *Hieracium* (Bicknell *et al.*, 2000). Among the diplosporous apomicts, independent inheritance of diplospory and parthenogenesis has been observed in the dandelion *Taraxacum* (van Dijk *et al.*, 1999) and in *Erigeron* (Noyes, 2000; Noyes and Rieseberg, 2000), whereas, Voigt and Burson (1983), reported the simple dominant inheritance of diplospory in *Eragrostis curvula*, the weeping lovegrass. Similarly the inheritance of diplospory in Eastern gamagrass (*Tripsacum dactyloides)* is reported to be simple and dominant (Leblanc *et al.*, 1995). There is evidence of segregation ratio distortion in some of these systems, often because the dominant factor (s) associated with apomixis also appears to confer gamete lethality, restricting its transfer to some gamete genotypes (Nogler, 1984b; Grimanelli *et al.*, 1998a; Roche *et al.*, 2001a; Jessup *et al.*, 2002). From these and other eariler studies, it is widely generalized that there is one locus or only a small number of loci, involved in the inheritance of apomixis in native systems.

Cytological and embryological analysis of the mother plant and screening of morphologically aberrant progenies are time-consuming and limited methods were traditionally employed for assessing the mode of reproduction. In the last few years, molecular markers have been used in several works for the identification of non-maternal offspring when individual DNA fingerprints reveal a deviation from the maternal profile. Genetic fingerprints have also been successfully used in the identification of hybrids in research programs involving apomicts. DNA fingerprinting helps in genotyping individuals of a mapping population. While ascertaining modes of reproduction, fingerprints can also be used to identify a marker for a trait. This involves genotype independent segregation analysis for the trait of interest. Various kinds of markers can be used for fingerprinting analysis. For developing trait specific fingerprint the plant material should be genetically more heterogeneous, while remaining genetically distant

to the trait of interest. Genotyping of an apomictic and sexual plant using DNA fingerprinting has been reported in very few cases (Huff and Bara, 1993). A simple bulked segregant analysis strategy can be used to identify markers closely linked to the apomictic reproduction.

Isolation of genes controlling apomixis can be approached mainly by two ways, mapping the gene (s) on a genetic linkage map and cloning the same by any of the various available methods, and direct isolation of the gene (s) exclusively expressed in apomictic plants using expression analysis. Besides, the study of enzymes and proteins differentially expressed in the apomictic and sexually reproducing plants can throw more light on the genetic control of apomixis leading to identification of functional genes that may have a role in apomixis. A possible strategy for direct isolation of gene(s) involved in apospory is by the method of subtractive hybridization. It is a powerful technique for isolating genes expressed or present in one cell population, but not in an another. cDNA can be prepared from population of mRNA from ovules of apomictic and sexual plants. Subtractive hybridization will allow isolation of differentially expressed cDNA that are specific to apomictic plants and are absent in the sexual plants.

Hence, the present study was conducted with the following objectives:

- To analyze isozyme and protein polymorphism in the mapping population.
- To fingerprint the apomictic and sexual segregants using bulked segregant analysis in *C. ciliaris*.
- To identify cDNA fragments associated with apomixis by subtractive hybridization.
- To analyze the putative genes/proteins using bioinformatics.

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

Apomixis

The term apomixis has, in the past, been used as a general term for any form of asexual reproduction in plants including vegetative propagation. This original definition has become more restricted and now covers only those asexual reproductive processes that, paradoxically, occur in the ovule of flowering plants-the structure that has evolved to carry out female sexual reproductive functions in angiosperms (Nogler, 1984a; Asker and Jerling, 1992). Some authors have chosen to use apomixis to describe all forms of asexual reproduction in plants, but this wider interpretation is no longer generally accepted. The current usage of apomixis is synonymous with the term "agamospermous" (Richards, 1997).

Apomixis has been described in >400 flowering plant taxa, including representatives of >40 families (Carman, 1997), and it is well represented among the monocotyledonous and eudicotyledonous plants. Several authors have noted a marked bias in the distribution of apomixis among angiosperms (Asker and Jerling, 1992; Mogie, 1992; Carman, 1997; Richards, 1997). Of the plants known to use gametophytic apomixis, 75% of confirmed examples belong to three families, the *Asteraceae*, *Rosaceae* and *Poaceae*, which collectively constitute only 10% of the flowering plant species. Conversely, although apomixis is known among the *Orchidaceae*, the largest flowering plant family, it appears to be comparatively uncommon among these plants. Some authors have postulated that the current patterns of distribution may reflect the predisposition of certain plant groups to the unique developmental and genetic changes that characterize apomixis (Grimanelli *et al.*, 2001b). This hypothesis appears intuitively attractive, but like many issues associated with apomixis, it remains conjecture until it is tested experimentally. Some of this bias also might relate to the ease of embryological examination in some plant groups or to data accumulated from embryological investigations associated with activities in crop improvement. With the exception of *apple* and *Citrus*, apomixis is not very common in agriculturally important crops. Because apomictically produced embryos are genetically identical to the female parent

plant, they are of obvious benefit to agriculture. If apomixis can be introduced into crop species, it can be an inexpensive way to perpetuate a given genotype, preserving even such characters as heterosis through successive generations via seed (Hanna and Bashaw, 1987). Apomictic plants could potentially provide a constant source of renewable seed capable of producing high-yielding food crops, an agricultural trait of great value, particularly for developing countries.

Potential value of apomixis in agriculture

Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform populations and perpetuates hybrid vigor through successive seed generations. Many agronomic advantages of apomixis can be envisioned: the rapid generation and multiplication of superior forms through seed from novel, currently underused germplasms; the reduction in cost and time of breeding; the avoidance of complications associated with sexual reproduction, such as pollinators and cross-compatibility; and the avoidance of viral transfer in plants that are typically propagated vegetatively, such as Potatoes (Hanna, 1995; Jefferson and Bicknell, 1995; Koltunow *et al.*, 1995a, Savidan, 2000a ,2000b). The value of these opportunities will vary between crops and between production systems. For farmers in the developed world, the greatest benefit is expected in the economic production of new, advanced, high-yielding varieties for use in mechanized agricultural systems. Conversely, for farmers in the developing world, the greatest benefits are expected in the release of robust, high-yielding varieties for specific environments, and improvements in the security of the food supply, and greater autonomy over variety ownership (Bicknell and Bicknell, 1999; Toenniessen, 2001).

However, apomixis is very poorly represented among crop species. The main exceptions to this appear to be tropical and subtropical fruit, such as mango, mangosteen and citrus, and tropical forage grasses such as *Panicum, Brachiaria, Dicanthium* and *Pennisetum*. It is possible that the low representation of apomixis among crops arose unintentionally from a protracted human history of selecting superior plants for future cultivation. Selection for change over a parental type would work against a mechanism such as apomixis that acts to maintain uniformity. The presence of the trait among tropical fruits and grass crops may be a reflection of this effect, because focused efforts to improve these crops are comparatively recent events (Bicknell *et al.*, 2004).

There are also few apomictic species of significant relatedness available for use in introgression programs, which may explain at least some of the difficulties experienced when attempts have been made to introduce apomixis into crops through hybridization. For example, major programs aimed at introducing apomixis into maize (Sokolov *et al.*, 1998; Savidan, 2000a) from the wild relative *Tripsacum dactyloides* have been under way now for decades, yet they have proven unsuccessful in terms of generating apomictic plants with agronomically acceptable levels of seed set. Difficulties also have been encountered in efforts to produce apomictic lines of hybrid millet (Morgan *et al.*, 1998; Savidan, 2001). Even if successful, it seems likely that introgression lines would provide limited flexibility in terms of practical capacity to manipulate apomixis in agricultural breeding systems. Current breeding efforts with apomictic crop species, such as the forage grasses *Brachiaria* and *Panicum* are frustrated by the need to use complex breeding strategies to accommodate the inaccessibility of the female gamete to generate hybrid progeny (Valle and Miles, 2001). We believe therefore that the best solution would be the introduction of apomixis into crops in an inducible format, permitting its use during seed increase but allowing for its silencing during hybridization. To achieve this, information will be required concerning the genes that control the trait, their interrelationship with sexual processes and the impact the trait might have on seed yield, viability and quality for a given plant (Bicknell *et al.*, 2004).

Sporophyte and gametophyte relationship

Sporophytic tissues of the ovule have maternal functions for the development of the seed, and contribute to the seed coat. Within the ovule primordium, the megasporangium produces the meiotic cells from which the female gametophyte is generated. The sporophytic cells that surround the female gametophyte express gene products required for female gametophyte development and function (Chaudhury *et al.*, 2001). Apparently meiosis and female gametophyte development are controlled by regulatory genes that may be deregulated in space and time in apomicts (Koltunow, 1993; Grossniklaus *et al.*, 2001b). Many events relevant to apomixis, such as the initiation of the egg cell and modified fertilization mechanisms are functions of the developing female gametophyte (Spillane *et al.*, 2001a). Grossniklaus *et al.*, (2001b) have identified mutants that were affected in female gametophyte development and double fertilization (Chaudhury *et al.*, 2001).

Apomeiosis: embryo development

Embryogenesis is the process in which the unicellular fertilized zygote divides and differentiates progressively into a bipolar structure called the embryo. The main body parts of the embryo include the apical meristem, hypocotyls, cotyledons, root and root meristem. Around the apical basal axis, a radial pattern is established and it contains the epidermis in the periphery and layers of vasculature and conductive tissues within (Chaudhury *et al.*, 2001). Embryogenesis in apomict occurs completely without the contribution of the paternal genome (Spillane *et al.*, 2001b) and an unreduced diploid egg develops into an embryo without fertilization (Chaudhury *et al.*, 2001).

Double fertilization

An important issue in sexual reproduction of an angiosperm's life cycle is seed development, which starts with the double fertilization phenomenon. In diploids, the female gametophyte is contained by the ovule. Contained within the ovule are a haploid egg cell at the micropylar end, a diploid central cell, two synergids adjacent to the egg cell, and three antipodal cells in the chalazal end (Chaudhury *et al.*, 2001). Pollination leads to fertilization of the egg cell and the central cell by sperm cells. The embryo is formed from the fusion of egg cell with the sperm cells whereas triploid endosperm results from the union of the maternal homodiploid central cell with a second sperm cell (Chaudhury *et al.*, 2001). This process occurs inside the maternal ovule tissues, which are surrounded by the diploid sporophytic layers of inner and outer integuments (Chaudhury *et al.*, 2001).

In contrast, apomictic plants by-pass or deregulate the sexual developmental program described above at various steps (Koltunow, 1993). Meiosis may be interrupted or absent resulting in the formation of an unreduced female gametophyte with the full complement of maternal chromosomes (apomeiosis). Fertilization may not occur and an autonomous embryo is produced (parthenogenesis). Endosperm development may be initiated autonomously or sexually. In the later case, embryo sac development or fertilization is often modified to adjust to a different genomic context (Grossniklaus *et al.*, 2001a; Naumova *et al.*, 2001a). With apomixis, only the female reproductive

program is altered, pollen formation is usually unaffected in apomicts (Spillane *et al.*, 2001b).

Parthenogenetic embryogenesis

Pseudogamy is the pollination and fertilization of a portion of the ovule. There are two types of pseudogamy, in the first; pollination and fertilization of the central cell are needed for parthenogenetic egg development. In the second type, initiation of embryo formation is precocious and independent of pollination and central cell fertilization, but fertilization-induced endosperm development is required for seed viability. Precocious embryo development also provides a means to avoid fertilization of the unreduced egg cell.

Apomictic *Citrus* species commonly produce many embryos that grow from the nucellar tissue of the ovule surrounding a fertilized female gametophyte. Digestion of the nucellus occurs as the sexually derived embryo and endosperm grow and expand within the nucellus, but the nuclear embryo initial cells are not digested, by this reason, seed of the apomictic *Citrus* are often highly polyembryonic (Garcia *et al.*, 1999). Successful fertilization of the sexually derived embryo sac is not required for the initiation and development of nucellar embryos in apomictic *Citrus*. In seedless and also in low-seeded *Citrus* varieties that exhibit high rates of female gametophyte abortion, the nuclear embryos initiate near a region of nucellar cell degradation in the chalazal region of the unfertilized ovule, quite distinct from the aborted female gametophyte (Chaudhury *et al.*, 2001).

Endosperm development

There are two hypotheses over the origin of endosperm:

1. One postulates that the endosperm forms from a twin embryo that assumed a growth support role.
2. The other hypothesis is that the endosperm results from the extended development of the megagametophyte, which may be promoted when the central cell is fertilized by the second male gamete (Chaudhury *et al.*, 2001).

Basic steps of endosperm development are conserved between monocots and dicots. Endosperm development in *Arabidopsis* and *Barley* consists of four stages:

syncytial, cellularization, differentiation and death. Endosperm has as a main function to nourish the embryo; in cereals, the endosperm is the site of storage reserves, but in species such as carrot, endosperm is thought as a source of signals involved in embryogenesis (Van Hengel *et al.*, 1998).

Endosperm development in apomicts can be autonomous or it may require pollination and fertilization (pseudogamy). Pseudogamy is typical for *Rosaceae* and *Graminae* families and it is the most common mode of the endosperm development in the apomictic plants (Chaudhury *et al.*, 2001). Some mutants affect endosperm development. For example, in the *fis* class of mutants *(mea, fis2, and fie)* diploid endosperm is formed autonomously without fertilization, thus endosperm development is suppressed by the wild-type function of these genes. Crosses between plants with different ploidy levels cause abnormal endosperm development, affecting successful hybridization (Chaudhury *et al.*, 2001). The maternal genome content of pseudogamous endosperm depends on the number of unreduced central cell nuclei fusing with the unreduced sperm cell nucleus. Autonomous endosperm production is rare in apomicts and is most common in the *Compositae* (Chaudhury *et al.*, 2001).

Apomixis-polyploids relationships

Naumova *et al.*, (2001a) enumerated two hypotheses to explain the prevalence of apomixis among polypliods. According to the hybridization-derived floral asynchrony theory, apomixis arose from asynchronous expression of duplicate sexual reproductive gene sets in hybrid or polyploid genomes. Nogler (1984a), Naumova *et al.*, (2001a), postulated that the alleles responsible for apomixis may act as, or are linked to, recessive lethal factors, thus they can only be transferred by a diploid or polypliod gamete.

Polyploid crops must pair and segregate closely related chromosomes correctly during meiosis in order to maintain genetic stability and fertility. Homologous chromosomes in polyploids, must accurately distinguish each other (Spillane *et al.*, 2001a). Additionally, facultative apomixis is associated with a high and irregular ploidy level or a high and variable level of heterozygosity (Barcaccia *et al.*, 1997). Thus, in polyploids apomixis is a difficult and precise process.

Gametophytic apomicts, irrespective of the mechanism they use, are almost invariably polyploids, yet sexual members of the same or closely related species are very commonly diploids (Asker and Jerling, 1992). The reason(s) for this association remains

unclear. It is, however, potentially a critical issue, because a frequently stated aim of current research is the installation of apomixis into diploid crop species. Three main theories have been forwarded. Some have proposed that the optimum expression of apomixis may be achieved only in conjunction with polyploid genome (Quarin *et al.*, 2001). Because rare diploid gametophytic apomicts have been reported (Asker and Jerling, 1992; Bicknell, 1997; Kojima and Nagato, 1997; Naumova *et al.*, 1999; Koltunow *et al.*, 2003a), polyploid does not appear to be absolutely required for the expression of the apomixis. In these examples, however asexual seed formation often was poor, so polyploidy may enhance the expression of apomixis in many systems rather than ensuring its expression per se. Some indications how this might operate come from *yeast* (Galitski *et al.*, 1999) *and Arabidopsis* (Lee and Chen, 2001), in which alterations in ploidy status are known to affect methylation and the expression of different alleles. Conversely, there are intriguing examples of apomixis being expressed in previously sexual plants after chromosome duplication (Nygren, 1948; Quarin *et al.*, 2001). However, there is some debate in these cases about the possibility of innate predisposition, because the plants used were sexual members of groups containing apomicts (Quarin *et al.*, 2001). Furthermore, the reverse has been described by Asker (1967), a sexual plant was recovered after the doubling of an apomictic biotype of *Potentilla argentea*. Finally, polyploidy has been induced in a large number of plants, and apomixis is reported very seldom in the products (Bicknell *et al.*, 2004).

An apparent interspecific hybrid origin also is a common feature among apomicts, and the combination of polyploidy and hybridity is believed to have resulted in allopolyploidy in many gametophytic apomicts (Ellerstrom and Zagorcheva, 1977; Carman, 1997, 2001; Roche *et al.*, 2001a). The action of tetrasomic inheritance in many systems, however also indicated the presence of autopolyploidy, or possibly segmental allopolyploidy, in these plants (Pessino *et al.*, 1999). Carman (1997, 2001) postulated that a combination of hybridity and polyploidy can lead to the disjunction of key regulatory events during critical stages of megasporogenesis, megagametogenesis and fertilization. This in turn may lead not only to apomixis but also to other unusual developmental events, such as polyspory and polyembryony. Through a comprehensive survey of the botanical literature, together with his own experimentation, Carman presented compelling evidences that there are associations between apomixis and these other phenomenon, that hybridity between related species has been key factor in the

formation of many apomictic complexes, and that different types of apomixis and related phenomena are all expected outcomes of a theoretical model based on the disjunction of a relatively small number of key regulatory events. Whether this is universally true, and whether it can be used to harness apomixis in crop species, are question remaining to be answered.

Roche *et al.*, (2001b) provided a refinement on Carman's hypothesis, suggesting that supernumerary chromatin may be the principal driver in this process. A hybrid origin, segmental allopolyploidy, and the activity of reproductive drivers all are reported characteristics of supernumerary chromatin biology (McVean, 1995). There is growing evidences for the presence of supernumerary chromatin in several apomictic species, and it is clearly involved in the inheritance of apomixis in the grasses *Pennisetum squamulatum* and *Cenchrus ciliaris* (Roche *et al.*, 2001a, 2001b).

Matzk *et al., (2003)* recently combined a flow cytometric seed screen for reproductive mode with chromosome counts and found that apomictic *Hypericum* and *Ascyreia* plants had a higher DNA content per chromosome than related sexual species. An increased genetic load mediated by transposon replication was postulated by Matzk *et al.*, (2003) as a mechanism by which the increased DNA content of apomictic *Hypericum* and *Ascyreia* might arise. We have evidence that at least four classes of transposons are present in *Hieracium* species, but the relative content of each in sexual and apomictic genomes has not been established (M.Tucker, T.Tsuchiya, R.Bicknell and A.M.Koltunow, unpublished results). Therefore, the enlarged genomes of apomicts might be more the consequence of asexual seed formation than its cause, and this may have contributed to the apparent involvement of supernumerary chromatin. This observation and conclusion, however, contradict the hypothesis that sexual species should have larger genomes than related asexual species (Wright and Finnegan, 2001), because sexual reproduction is thought to favor the spread of mobile elements between individuals of a population and asexuality is thought to prevent interindividual transfer (Hickey, 1982; Matzk *et al.*, 2003).

Sexual -apomictic interrelationships

Most apomicts are facultative, that is both apomictic and sexual reproduction occurs at different levels and even within the same plant. Obligate apomicts are those plants in which a sexual reproduction pathway is absent (Spillane *et al.*, 2001b). There

are species, such as *Poa pratensis*, in which its reproductive behavior ranges from nearly obligate apomixis to complete sexuality (Barcaccia *et al.*, 1997).

Models of apomixis

Apomixis can occur due to heterochronicity or heterotocipity of the sexual developmental process of reproduction. Heterochroinicty refers to mistakes in the timining of developmental processes, for example; megagametogenesis occurs before megasporogensis is completed or embryogensis starts prior to fertilization (Spillane *et al.*, 2001b). Causes of the heterochronic development can be perturbation of developmental checkpoints or changes in gene expression due to polyploidization (Grossniklaus, 2001a). Heterotocipity is related to mistakes in the location where developmental events occur. It may be due to deregulation of cell fat especification (Grossniklaus, 2001a). The formation of the embryo initials from integumentary or nucellar cells (adventitious embryony) and the formation of a gametophyte from nucellar cells (apospory) are examples of heterotocipity effects in apomicts (Spillane *et al.*, 2001b).

Types of apomixis

A defined sequence of events must be completed to result in the generation of a fertile and genetically unique seed that is the end product of sexual reproduction in angiosperms. This sequence comprises of the following events: megaspore mother cell differentiation from the nucellus, megaspore production by meiosis (megasporogenesis), megaspore selection, embryo sac development by mitotic processes (megagametogenesis), embryo sac maturation, double fertilization and endosperm and embryo formation. In sexually reproducing plants, the absence or disruption of any one of these steps usually results in cessation of the developmental program, and a viable seed is not produced.

In contrast to sexual reproduction, apomictic processes can completely omit some of the events in this sequence and still produce a fully formed, viable embryo within the confines of the ovule. Cytological studies have revealed that apomictic processes always deviate from sexual reproduction in more than one respect (Asker, 1979, 1980; Nogler, 1984a; Asker and Jerling, 1992). These developmental differences occur in commonly

identifiable combinations, such that apomictic processes have often been divided into three mechanisms, termed diplospory, apospory and adventitious embryony.

These apomictic mechanisms differ from one another in the time at which the process is initiated during ovule development relative to the normal sexual pathway. Diplospory and apospory result in the formation of a megagametophytic structure without meiotic reduction, and the embryo develops from a cell inside this unreduced megagametophyte. Diplospory and apospory are, therefore commonly referred to as gametophytic apomictic processes (Nogler, 1984a; Asker and Jerling, 1992). By contrast, adventitious embryony is initiated late in the ovule development and usually occurs in mature ovules. Embryos are initiated directly from individual cells in ovule tissues that are external to a sexually derived megagametophyte. Therefore adventitious embryony has been described as sporophytic apomixis (Nogler, 1984a; Asker and Jerling, 1992). Most plants with gametophytic apomixis are polyploid; however, genera with adventitious embryony are commonly diploids (Asker and Jerling, 1992).

1. Gametophytic apomixis

In this type of apomixis embryo sacs are produced from unreduced initial cells. The egg cell develops parthenogenetically, giving rise to embryos that give rise to plants resembling the maternal parent. Gametophytic apomixis may be obligatory (predominantly apomictic) or to some extent combined with sexuality (facultative). It is strongly associated with polyploidy and is rare in diploids. Gametophytic apomixis is further divided into two categories, diplospory and apospory (Crane, 2001).

A). Diplospory

In diplospory unreduced embryo sacs are formed from megaspore mother cells (MMCs) by circumvention of meiosis. The embryo develops parthenogenetically from the unreduced egg, and the endosperm develops autonomously (without fertilization) from the unreduced polar nuclei. Thus, pollination in the diplosporous apomicts is not necessary. Koltunow (1993) classified diplospory into two categories: meiotic and mitotic diplospory. Four types of diplosporic embryo sac development have been described (Nogler, 1984a). These have been named after the genera in which they were first described.

i). *Taraxacum* type

The MMC initially enters meiotic prophase, and normal chromosome pairing does not take place due to asynapsis. The univalents are scattered over the spindle at metaphase I. A restitution nucleus is formed after first meiotic division, which subsequently divides mitotically to form a dyad with somatic (2n) chromosome number. Further mitotic divisions result in eight–nucleate embryo sac. (Cooper and Brink, 1949; Richards, 1997) This type of apomixis occurs in some of the genera of Compositae and in *Arabis* and *Paspalum species* (Nogler, 1984a).

ii) *Ixeris* type

The MMC undergoes asyndetic meiotic prophase resulting in restitution nucleus. This is followed by a division similar to the second meiotic division except that it is not associated with cytokinesis. Two mitotic divisions of the unreduced nuclei result in eight-nucleate embryo sac (Nogler, 1984a).

iii). *Antennaria* type (mitotic diplospory)

MMC does not go through meiosis and functions as an unreduced megaspore. After a long interphase, it begins to divide mitotically which results in the formation of typical eight-nucleate embryo sac. This type of diplospory has a wider taxonomic distribution (Nogler, 1984a).

iv). *Allium* type

In *Allium* type of diplospory, pre-meiotic chromosome doubling is the cause of unreduced embryo sac formation (Hakanson and Levan, 1957). Chromosome number is doubled by a pre-meiotic endomitosis and a normal meiosis results in a tetrad of unreduced nuclei. Two subsequent mitosis in the chalazal dyad result in eight nucleate embryo sac. Among diplosporous species, *Allium nutans* and *A.odorum* have a disporic embryo sac development of *Allium* type (Nogler, 1984a).

B). Apospory

In apospory, unreduced embryo sacs arise from somatic cells in the ovule. The somatic cells of the ovule from which embryo sacs develop belong to the nucellus.

Several cells of the nucellus may start aposporous development but usually only one of them gives rise to mature embryo sac. Apospory is initiated after MMC differentiation. The megaspore degenerates and the aposporous embryo sac occupies the position near the micropylar end of the ovule. The embryo develops parthenogenetically from the unreduced egg, but pollination is required for the development of endosperm. Apospory is common in large number of apomicts in the grass family *(Pennisetum, Cenchrus, Poa)*. In *Poaceae*, apospory is the predominant form of apomixis (Asker and Jerling, 1992). Apospory is of two types.

i). *Hieracium* type

In this type, eight-nucleate bipolar embryo sac is formed. The unreduced embryo sac has its origin in a somatic cell confined to the center of the nucellus adjoining the chalazal pole of the MMC. Aposporous initials are recognized by the growth and enlargement of the nucleus and nucleolus and by vacuolation causing compression and degeneration of able sexual megaspore (Nogler, 1984a).

ii). *Panicum* type

In this type, four nucleate monopolar embryo sac is formed. Compared to the *Hieracium* type, the *Panicum* type is characterized by the absence of the initial polarization in the progenitor cell of the embryo sac and by the vacuolation of the chalazal end of the cell. The spindle of the first mitotic division lies crosswise at the micropylar end, and a second mitosis leads to the formation of four free nuclei. Later, these nuclei organize into the female gametophyte consisting of a three-celled egg apparatus and a single polar nucleus, antipodals are absent. Thus the four nucleate monopolar embryo sacs formed are unreduced (Koltunow, 1993; Czapik, 1994). Although the four- nucleate embryo sac is the rule in the *Panicum* type apospory, some exceptions have been reported. In certain cases, unreduced embryo sacs are bipolar and eight-nucleates (Nogler, 1984a).

2. Adventitious embryony

In adventitious embryony, embryos develop from cell in the tissues external to a sexual embryo sac. Adventitious embryos arise from individual cells of the two different somatic tissues, nucellus or integument (Lakshmanan and Ambegaokar, 1984).

Adventitious embryony is purely a sporophytic form of agamospermy. It usually occurs in the presence of normal sexual reproduction and results in polyembryony. It is initiated late in ovule development and usually occurs in mature ovules. Embryos are initiated directly from the individual cells and are not surrounded by megagametophytic structure or embryo sac. This is in contrast to sexual, aposporous and diplosporous reproduction, in which the cell that develops into the embryo is part of a megagametophyte like structure (Koltunow, 1993). Adventitious embryony commonly occurs in diploid species. It is common in citrus and mango.

Apospory in buffelgrass

Buffelgrass is an important perennial forage and range grass throughout the semi-arid tropics (Bogdan, 1977). The species reproduces predominantly by aposporous apomixis, but sexual genotypes have been identified (Fisher *et al.*, 1954; Snyder *et al.*, 1955; Bashaw, 1962; Bray, 1978; Sherwood *et al.*, 1980, Gupta *et al.*, 2001). Although the number of chromosomes have been reported to range from 32 to 54 for buffelgrass (Fisher *et al.*, 1954; Hignight *et al.*, 1991; Visser *et al.*, 2000), most genotypes are tetraploids (2n=4x=36). Cytological observations have indicated that the 36-chromosome types are segmental allotetraploids with 10–14 bivalents and 2–4 quadrivalents at diakinesis (Fisher *et al.*, 1954; Snyder *et al.*, 1955). Even though spindle formation and cytokinesis appear normal, 1–5 lagging univalents are occasionally observed at anaphase I (Fisher *et al.*, 1954; Snyder *et al.*, 1955; Hignight *et al.*, 1991). Thus, disomic, tetrasomic, and distorted inheritances are possible. Segregation studies of apomixis in buffelgrass have reported both disomic (Taliaferro and Bashaw, 1966) and tetrasomic (Sherwood *et al.*, 1994) inheritances, with potential for recessive lethality.

Inheritance of apomixis

Apomictic species are poor subjects for genetic study. The data from most crosses between apomictic and sexual individuals have not been conclusive. Some of the difficulties are due to the complex polyploid nature of the apomictic species. The first known study of inheritance in an apomictic plant was unknowingly conducted by Gregor Mendel (1869) on *Hieracium*, ironically selected to assist in corroborating his laws of inheritance (reviewed by Correns, 1905; Nogler, 1994). It is not known how many

crosses Mendel conducted on *Hieracium* because most of the data have been lost, but extrapolations from the information that is available indicates that he performed many thousands of crosses over a period of >10 years. By direct contrast to his observations in pea, the *Hieracium* F1 hybrids showed extensive segregation, whereas the F2 hybrids did not segregate and uniform progeny were obtained consistently. In correspondence with Nageli, a *Hieracium* specialist (July 1870), Mendel noted the "almost opposed behavior" in the two systems "both (of which represented) the emanation of a higher universal law." By the turn of the 20th century, apomixis was a known phenomenon in plants, and Ostenfeld (1904, 1906, 1910) returned to the study of inheritance in *Hieracium*. He conducted several cross combinations, including repetitions of Mendels work, and along with Rosenberg (1906, 1907), correctly noted the expression of apomixis in the genus. It had taken almost 40 years to explain Mendel's data.

Genetic studies largely depended on crosses and recombination events neither of which is easily obtained in apomicts. However, because most apomicts produced normal, reduced pollen, the inheritance of apomixis could be investigated by analyzing the segregation ratios in crosses with related sexuals. Such analyses are difficult because gametophytic apomicts are almost without exception polyploids, causing complex modes of inheritance. An assessment of the breeding system in the hybrids requires cytological observations or, at least, time-consuming progeny tests. Variation in the expressivity of apomixis may create an additional complication. Moreover, much of the earlier work was done on the *Rosaceae*, which are extremely difficult to analyze because the multiple MMCs formed in sexuals make the distinction between reduced and unreduced embryo sacs difficult. As a consequence, for many years the genetics of apomixis seemed unclear, complex, and idiosyncratic. However, since the end of the 1970s, a clear general pattern in the inheritance of various types of gametophytic apomixis has emerged, first in the *Ranunculaceae* and *Poaceae* and later in the *Compositae*.

The inheritance of gametophytic apomixis has since been reported to be associated with the transfer of either single locus or a small number of loci in most of the systems studied to date. In the aposporous grasses *Pennisetum* (Sherwood *et al.*, 1994), *Panicum* (Savidan, 1983), and *Brachiaria* (Valle *et al.*, 1994), apomixis is reported to be simply inherited with the trait conferred by the transfer of a single dominant factor. Simple dominant inheritance also has been reported for apospory in the dicotyledonous genera *Ranunculus* (Nogler, 1984b) and *Hieracium* (Bicknell *et al.*, 2000). Among the

diplosporous apomicts, independent inheritance of diplospory and parthenogenesis has been observed in the dandelion *Taraxacum* (van Dijk *et al.,* 1999) and in *Erigeron* (Noyes, 2000; Noyes and Rieseberg, 2000), whereas Voigt and Burson (1983), reported the simple dominant inheritance of diplospory in *Eragrostis curvula,* the weeping lovegrass. Similarly the inheritance of diplospry in Eastern gamagrass (*Tripsacum dactyloides)* is reported to be simple and dominant (Leblanc *et al.,* 1995). There is evidence of segregation ratio distortion in some of these systems, often because the dominant factor (s) associated with apomixis also appears to confer gamete lethality, restricting its transfer to some gamete genotypes (Nogler, 1984b; Grimanelli *et al.,* 1998a; Roche *et al.,* 2001a; Jessup *et al.,* 2002).

Using more suitable apomictic species and focusing on one element of apomixis, apomeiosis, pioneer studies by Nogler in the buttercup species *Ranunculus auricomus* and by Savidan in the grass *Panicum maximum* indicated that apospory in these two species segregated as a single dominant Mendelian factor (Savidan, 1982; Nogler, 1984b,). Subsequent investigations showed that both apospory and diplospory in other species also fitted this segregation model (Table 1). Although a dominant Mendelian factor can represent any genetic constitution from a single gene to an entire chromosome (e.g., mammalian sex determination), these observations often were taken as evidence for monogenic inheritance. According to this model, apomictic plants possess the simplex genotype *Aaaa,* carrying in addition to the dominant apomeiosis allele *A* several recessive alleles for sexual reproduction. Apomictic plants thus carry the potential for sexual reproduction, but in a more or less repressed state, because of the presence of the dominant apomixis factor. Limited penetrance of the apomixis factor explains the occurrence of facultative apomixis. The presence of recessive sexual alleles explains how a cross between two facultative apomicts can generate abundant purely sexual offspring. Although the occurrence of apomixis fits this model, the degree of apomixis often is dependent on environmental conditions (Nogler, 1984a) and/or on modifier genes (Bicknell *et al.,* 2000). These as yet unspecified factors need further investigation in the future. Moreover, it remains to be determined whether this general model also applies to many apomicts in the *Rosaceae*.

Table1. Inheritance of elements of gametophytic apomixis (apomeiosis and parthenogensis) in memebers of the *Ranunculuaceae, Poaceae and Compositae*

Species	Apomeiosis type	Family	Inferred genotype	Most closely linked molecular marker	Evidences for suppression recombination	Reference
Apomeiosis						
Ranunculus auricomus	Apospory	*Ranunculus*	Aaaa	-	-	Nogler, 1984b
Panicum maximum	Apospory	*Poaceae*	Aaaa	-	-	Savidan, 1982
Pennisetum squamulatum	Apospory	*Poaceae*	Aaaa	0 cM	Yes	Ozias-Akins *et al.*, 1998
Brachiaria decumbers	Apospory	*Poaceae*	Aaaa	1.2 cM	Yes	Pessino *et al.*, 1998
Paspulum simplex	Apospory	*Poaceae*	Aaaa	0 cM	Yes	Pupilli et al., 2001
Hieracium piloselloides	Apospory	*Compositae*	Aaa	-	-	Bicknell *et al.*, 2000
Hieracium aurantiacum	Apospory	*Compositae*	Aaa	-	-	Bicknell *et al.*, 2000
Tripsacum dactyloides	Diplospory	*Poaceae*	Aaaa	0 cM	Yes	Grimanelli *et al.*, 1998a, 1998b
Erigeron annuus	Diplospory	*Compositae*	Aaa	0 cM	Yes	Noyes and Rieseberg, 2000
Taraxacum offcinale	Diplospory	*Compositae*	Aaa	4.4 cM		Van Dijk *et al.*, 2004
Parthenogenesis						
Poa pratensis	Apospory	*Poaceae*	Pppp	6.6cM		Barcaccia *et al.*, 1998
Erigeron annuus	Diplospory	*Compositae*	Ppp	7.3 cM	No	Noyes and Rieseberg, 2000

Apomeiosis locus is located in a recombinationally suppressed region

The segregation model described above has been supported and refined by the isolation of molecular markers that are linked to the presumed apomixis loci in several species (Table 1). In all cases in which it has been critically tested to date, a strong suppression of recombination around the apomeiosis locus has been found. For instance, strict cosegregation with apomeiosis of many more molecular markers than expected was found in aposporous *Pennisetum squamulatum* (Ozias-Akins *et al.*, 1998) and diplosporous *Erigeron annuus* (Noyes and Rieseberg, 2000). In *Brachiaria decumbens* (Pessino *et al.*, 1998), *Tripsacum dactyloides* (Grimanelli *et al.*, 1998a), and *Paspalum simplex* (Pupilli *et al.*, 2001), comparative mapping with maize or rice markers showed a lack of recombination in the region associated with the apospory locus. Markers that were spread over a region ranging from 15 to 40 centimorgans in the sexual relatives strictly cosegregated in these apomicts. Repression of recombination could frustrate map-based cloning efforts because closely linked markers may be at great physical distances from the apomixis loci.

Because suppressed recombination occurs in both dicot and monocot species, it may be a general characteristic of apomeiosis loci. This could be related to their function as observed in other complex loci containing several genes involved in a common process (coadapted gene complexes), such as the heterostyly supergene in *Primula* (Ernst, 1936), the self-incompatibility (*S*) loci in *Brassica* (Lewis, 1962; Awadalla and Charlesworth, 1999), the mating-type locus in *Chlamydomonas* (Ferris and Goodenough, 1994), and the major histocompatibility locus in humans (O'hUigin *et al.*, 2000). Alternately, it could be an evolutionary by-product of long-term asexual reproduction (Judson and Normark, 1996; Welch and Meselson, 2000). In *Pennisetum* species, markers that are linked to apospory in the apomicts could not be detected by hybridization in sexual relatives (Ozias-Akins *et al.*, 1998; Roche *et al.*, 1999), indicating that the apomicts were either hemizygous for the apomixis locus (*A*---) or that the alleles were highly divergent (*A a' a' a'*), as was observed for the *Brassica S* locus (Boyes *et al.*, 1997; Suzuki *et al.*, 1999).

One master apomixis gene or several independent apomixis genes

Apomictic development deviates from the sexual pathway in apomeiosis, parthenogenesis, and often endosperm development (autonomy, altered embryo sac development, or altered fertilization). Are these elements of apomixis all controlled by a single gene or by several genes? In the pioneering studies on *R. auricomus* and *P. maximum*, parthenogenesis was strictly associated with apospory. Hence, apomixis as a whole inherited as a single mendelian trait (Savidan, 1982; Nogler, 1984b). Similarly, in *Hieracium piloselloides*, all three elements have inherited as a single genetic trait (Bicknell *et al.*, 2000). In these species, apomixis could be regulated by a single master regulatory gene controlling all elements or by a gene complex of several tightly linked genes that are recombinationally locked. In other species, however, crosses between sexuals and apomicts have yielded progeny combining elements of both the sexual and the apomictic developmental pathways. In *Taraxacum officinale*, hybrids were recovered that displayed diplospory and autonomous endosperm development but that lacked parthenogenesis (van Dijk *et al.*, 1999). Such "apomixis recombinants" also have been reported in *Poa pratensis* (Matzk *et al.*, 2000), which suggested that the elements of apomixis in these species are regulated by different genes. In *Erigeron annuus*, separate genes for diplospory (*A*) and parthenogenesis (*P*) have been mapped genetically (Noyes and Rieseberg, 2000). Apomicts in this species carry the simplex genotype for both genes (*Aaa* and *Ppp*). In contrast to the diplospory *A* locus, no suppression of recombination was observed around the parthenogenesis *P* locus (Table 1). In light of these new findings, it is likely that coadapted gene complexes are present in those species in which all elements of apomixis cosegregated.

Segregation distortion of apomixis loci

As mentioned above, gametophytic apomicts are usually polyploid, whereas related sexuals are diploid. Is gametophytic apomixis incompatible with diploidy? Again, the pioneering work on *R. auricomus* by Nogler appears to have general relevance. Nogler showed that diploid offspring that developed parthenogenetically from reduced diploid egg cells of tetraploid apomicts (dihaploids) or diploids produced through anther culture were able to reproduce apomictically (Nogler, 1982). This shows that apomixis

and diploidy are not incompatible, a finding that has been confirmed in several other species (Bicknell, 1997; Kojima and Nagato, 1997). However, what matters is the origin of the diploid offspring, because zygotic diploids derived from the fusion of haploid egg cells and haploid sperm never reproduced apomictically in *Ranunculus*. Nogler hypothesized that the apospory (*A*) locus was recessive lethal in the gametes. Consequently, the *A* locus could be transmitted via diploid gametes to generate polyploid apomicts but not via haploid gametes to generate diploid apomicts. It is also possible that mutations closely linked to the *A* locus cause haploid gamete nonfunctionality. The net result is that haploid gametes carrying the *A* locus do not contribute to offspring production, resulting in segregation distortion of the *A* locus.

More recently, additional evidence has been obtained for segregation distortion of apomixis loci in other plant species, such as *Tripsacum dactyloides*, *Pennisetum squamulatum*, and *E. annuus* (Grimanelli *et al.*, 1998b; Ozias-Akins *et al.*, 1998; Noyes and Rieseberg, 2000). Transmission studies of markers linked to apomixis loci in *E. annuus* indicated different causes of nontransmission, the parthenogenesis locus *P* in *E. annuus* was not transmitted because of selection against haploid gametes, as was observed for the *A* locus in *R. auricomus*. The diplospory locus *A*, in contrast, was not transmitted because of meiotic drive. In these triploid apomicts, the nondiplospory chromosomes seem to pair preferentially, leaving the diplospory chromosome as a univalent that always ends up in a diploid pollen grain (Noyes and Rieseberg, 2000). In *Hieracium piloselloides*, different crossing schemes indicated that apomixis can be transmitted via both haploid and diploid gametes, but post-zygotic lethality rather than segregation distortion causes the absence of apomixis in diploids (Bicknell *et al.*, 2000).

Techniques for identifying apomicts

Screening techniques

Various techniques involving cytological, genetic and histochemical examinations are used to screen for apomixis and to identify the apomictic mechanism. Some of the commonly used techniques are described below:

Morphology

Uniformity of progeny from heterozygous or cross-pollinated parents is the best indication of apomixis. Occurrence of maternal phenotypes in crosses is another indication of apomixis. Screening for apomixis can be expedited if pollen from a dominant marker stock is used. Production of maternal progeny with recessive phenotype would indicate an apomictic mode of reproduction. The technique would be more efficient if the dominant marker is identifiable at seedling stage. High seed set in the progenies of aneuploid plants or wide–cross derivatives is another indication of apomixis. Occurrence of multiple stigma and multiple ovules per floret may be due to apomictic mode of reproduction (Hanna *et al.*, 1970; Hanna, 1991). Consistently high frequency of twin seedlings in the progenies is another indication of apomixis. Multiple seedlings per seed can be due to development of multiple aposporous embryos in an ovule or facultative apomixis where embryo develop in both sexual and apomicitic embryo sac, and, development of adventitious embryos in addition to the sexual embryo.

Cytological techniques

Embryo sac analysis is one of the commonly used techniques for studying apomixis. Cytological analysis of developing embryo sac is performed at different stages from initiation of MMC to the formation of mature embryo sac. In apospory, embryo sac develops from a somatic cell. Multiple aposporous embryo sac initials are observed, which may be clustered around the megaspore. One of the aposporous cells matures into an embryo sac that has four nuclei, three of which form the egg-apparatus and the fourth serves as a polar nucleus. Thus lack of antipodals in the embryo sac is a diagnostic feature of apospory.

The pistil-clearing technique (Young *et al.*, 1979,Crane and Carman, 1987) is widely used for examination of the embryo sacs. Pistil clearing methods using aromatic esters greatly reduce the time needed to prepare the sample for examination. The method consists of fixing pistils at the time of anthesis in formalin- acetic acid-alcohol (FAA) consisting of 70% ethanol, glacial acetic acid, 37% formaldehyde (18:1:1) and passing pistil through alcohol series and clearing with methyl salicylate and examining under phase contrast microscopy.

Another distinguishing feature of sexual embryo sacs is the deposition of callose on the cell walls. In sexual plants the walls of the MMC (or megasporocyte), the tetrad of megaspore and degenerating megaspore are marked by the temporary accumulation of callose. It is lost from the walls of the selected megaspore during its expansion and is absent once the mitotic events of embryo sac development initiate. Apomictic embryo sacs are devoid of a callose layer. Differences in callose deposition during gametophyte development in sexual and apomictic plants have been noted, and in case of *Tripsacum*, this has been used as a screening tool to identify apomicts (Leblanc and Mazzucato, 2001). Callose fluorescence is used in combination with pistil clearing to detect diplosporous embryo sac development. Carman *et al.*, (1991), compared the embryo sac of sexual *Elymus scabrus* and diplosporous *E. rectisetus*. Callose accumulated in and around the cell walls of embryo sacs of *E. scabrus* but was absent in those of *E. rectisetus*. Peel and Carman (1992), combined the pistil clearing technique with callose fluorescence to screen rapidly for apomixis. A sucrose clearing solution (2.46M sucrose, 1.36mM aniline blue, 50mM K2HPO4, pH 9.5) induced excellent callose fluorescence of embryo sac walls. This technique is being used at, the International Maize and Wheat Improvement Center (CIMMYT) to screen *Tripsacum* germplasm and the breeding materials from Maize- *Tripsacum* crosses. Callose was completely absent from aposporous initial cells in *Poa pratensis*, *Pennisetum* (Peel *et al.*, 1997), *Brachiaria* (Dusi and Willemse, 1999) and *Hieracium* (Tucker *et al.*, 2001).

Histological techniques

This technique is employed to study the embryo sac development by examining histological serial sections of ovules. Female florets at different stages of maturity are collected and fixed in FAA for 24 hours and are then transferred to 70% ethanol. Pistils are dissected and dehydrated using the method of Young *et al.*, (1979). The pistils are then embedded in parafilm, sectioned at 10µm and stained with safranin O- fast green. Sherman *et al.*, (1991) analyzed embryo sac development by examining histological section of ovules of *Tripsacum dactyloides* to detect failure of meiosis. In buffel grass Bashaw and Hignight (1990) found proembryos in 15% of the ovules collected before pollination, but none of them contained endosperm. Apparently pollination is necessary for endosperm development, and probably also precedes the initiation of embryo development in most ovules.

Biochemical techniques

Apomictic progenies will be genetically uniform and will be identical to the mother plant. Hence various biochemical and molecular techniques can be employed to distinguish apomictic and sexual progenies. Isozyme markers can be used to study genetic variation and to detect the presence of apomixis. Roy and Rieseberg (1989) detected the apomictic breeding behavior of *Arabis holboelli* through enzyme electrophoresis. Morphology revealed no genetic variation within families, yet interfamily diversity was quite high. Kojima *et al.*, (1991) estimated the degree of apomixis by electrophoresis analysis of leaf esterase in progenies obtained from crosses of six cultivars of *Allium tuberosum*. More than 90% of seedlings showed the same zymogram as their pistillate parent, the remaining seedlings showing hybrid zymograms indicating 90% apomixis in each of the cultivars. In another study isozyme polymorphism of the sexual pool was higher (69%) compared to apomictic pool (Assienan and Noirot, 1995). Gustine. *et al.*, (1996), observed polymorphism in 12 out of 22 isozyme systems tested using leaf extracts where none of the isozyme co-segregated with apomixis.

Molecular techniques

With the advent of newer molecular biological techniques a variety of approaches have been available to study genetic variation among individuals at the DNA level. These methods offer advantages, quick, easy and precise such as RAPD, RFLP and AFLP. If the genes for apomixis can be tagged with molecular marker such as RFLPs, breeding materials can be screened for apomixis through linked molecular markers. Ozias-Akins *et al.*, (1993) found two molecular markers (UGT 197 and OPC-04) that cosegregated with apomictic mode of reproduction, in the cross between apomictic aneupliod plant derived from a trihybrid of two wild species of *Pennisetum* and sexual Pearl millet. Hanna *et al.*, (1993), used these molecular markers to screen BC4 progenies derived from the cross of apomictic BC3 plants and sexual Pearl millet. In a random sample of eight BC4 progenies examined through molecular markers, five were apomictic and three were sexual. Embryo sac analysis of the eight plants confirmed the results of molecular

analysis. The value of molecular markers, tightly linked to the apomictic genes, in breeding program is thus obvious.

Development of mapping population for apomixis

A proper mapping population that cosegregates with the desired trait is a prerequisite for any genetic and molecular studies. Gustine *et al.*, (1996), grew eight half-sib progeny lines from seed of the open pollinated *Cenchrus ciliaris* B-2s[sexual lines B-5-2, B-5-7, B-11-7, B-14-8 and B-18-7 (aaaa); aposporous lines B-1-9, B-12-9 and B-21-7 (Aaaa)]. A full-sib buffel grass *(Cenchrus ciliaris)* population, derived from crossing a highly heterozygous, sexual genotype (90C48507) with a highly heterozygous, apomictic genotype (PI 409164), was the source of 87 F1 hybrids included in mapping population (Jessup *et al.*, 2002). Ozias-Akins (1998), developed F1 mapping population of 309 individuals from a cross of *Pennisetum glaucum* and *P.squamulatum.* Albertini *et al.*, (2001a), had developed F1 mapping population of 48 individuals from controlled crosses between completely sexual clones (S1, S2, S4) and highly apomictic genotypes (RS-9, RS-18, RS-20) in *Poa pratensis* (Matzk, 1991; Barcacia *et al.* 1997). Martinez *et al.*, (2003), had developed a total of 290 F1 hybrids, by the controlled crosses in *Paspalum notatum*.

Identification of molecular marker for apomixis

Apomixis needs to be easily assessed and quantified in a manner that preferably avoids the application of tedious histological methods or progeny tests. Rapid and accurate quantification of the character is necessary during crossing experiments between sexual and apomictic plants (Koltunow *et al.*, 1995a). Means of readily determining reproductive mode of sexual-by-apomictic hybrid progenies are also essential for efficient breeding programs of apomicts (Hanna, 1995). The recent developments in molecular marker technologies offers the prospect of rapid screening for the trait, if an appropriate linked marker can be found. Molecular markers also raise the possibility to develop comparative studies on genomic structure and could provide a tool for the isolation of gene(s) that governs the trait through positional cloning, if they were found linked close enough to the target locus to vindicate such a strategy.

The identification of the genes involved in apomixis appears to be tractable, because in most of the native apomictic systems under study, only a small number of loci have been determined to be critical for the inheritance of the trait. In most cases, a map-based approach has been taken to attempt the cloning of the sequence involved. The first and most comprehensive mapping efforts in apomicts have been reported from group working in grass species, most of which are relatives of important cereals crops.

a). Identification of marker linked to Apospory

The earliest studies reporting the identification of molecular markers for apospory were carried out by Ozias- Akins *et al.*, (1993) in complex interspecific hybrids of *Pennisetum*, produced by crossing induced tetraploid pearl millet *(Pennisetum glaucum)* (2n=4x=28), a wild apomictic species *(Pennisetum squamulatum)* (2n=6x=54) and a bridging species *(Pennisetum purpureum)* (2n=4x=28). The search for molecular markers linked to apomixis was performed on backcross 3 (BC3) clonal line and the four cultivars or accessions in its pedigree. Out of the several markers reported, two appeared to be outstanding, RFLP fragment UGT197 and RAPD marker OPC-04. Both detected a marker in BC3 that was shared only with the apomictic parental accessions of *P. squamulatum*. UGT197 did not hybridize at all with the non–apomictic genotypes in the pedigree of BC3. UGT197 and OPC-04 were always present in obligate apomicts. In a subsequent work (Lubbers *et al.*, 1994) the occurrence of markers detected by UGT197 and OPC-04 in a range of 29 accessions of *Pennisetum* showed that UGT197 was more consistently associated with the trait. RAPD markers were also used to produce a map of apospory-region in *Pennisetum ciliare* (Gustine *et al.*, 1997). Pairwise linkage analysis of new markers together with the STS marker (UGT197) previously reported by Ozias-Akins *et al.*, (1993) and Lubbers *et al.*, (1994) provided a preliminary map of the chromosome bearing the apospory gene.

A very interesting feature regarding the structure of the genomic region controlling apospory in *Pennisetum squamulatum* was recently reported by Ozias-Akins *et al.*, (1998). After producing 4000 total RAPD markers, 12 were found to strictly cosegregate with apomixis. The genomic region detected by these markers proved to be absent in the genome of the sexual plants when assayed in genomic Southern blots. The apomixis gene(s) appeared to be present in a hemizygous condition, where recombination is repressed, which was called ASGR (Apospory Specific Genomic Region). The

dominant factor associated with the inheritance of apomixis in the aposporous plus pseudogamous grass species *Pennisetum squamulatum* appears to be hemizygous, because no equivalent region has been found in sexual biotypes (Ozias-Akins *et al.*, 1993, 1998; Roche *et al.*, 1999, 2001a). Intriguingly, the same region was found in the apomictic relative *Cenchrus ciliaris*, also as a hemizygous region (Roche *et al.*, 1999, 2001a). Efforts to isolate this region have revealed its complex nature. Lack of recombination near apomixis locus could again (similar to the *Tripsacum* example) play a role in maintaining inheritance of the trait by avoiding dispersion of a group of genes that are coupled in function (Grimanelli *et al.*, 1998a). Even when at first sight these results may appear analogous to those presented for *Tripsacum*, a substantial differences should be noted; in *Tripsacum* lack of recombination involves markers that map in a region present in both diplosporous and sexual plants. In *Pennisetum* lack of recombination occurs in a genomic segment that is unique to aposporic plants. After the isolation of BACs corresponding to these markers, it became apparent that this region of suppressed recombination spanned at least 50 Mbp in *P. squamulatum* (Roche *et al.*, 2002) and may be considerably larger. Using the corresponding BAC clones as probes, Goel *et al.*, (2003) demonstrated that the apospory-specific genomic region localizes to a single short arm of a *P.squamulatum* chromosome.

Brachiaria hybrids were also assayed to find molecular markers linked to apospory (Pessino *et al.*, 1997). Previous test provided strong evidence on single gene inheritance of the reproductive mode (Tohme *et al.*, 1996a). Bulked segregant analysis (BSA) and a systematic maize-based scanning of hybrid genome allowed the identification of markers linked to apospory that map to the distal part of the short arm of maize chromosome 5. RAPD analysis yielded one more marker (OPC-04) significantly linked to the trait (Pessino *et al.*, 1997), which had been previously reported as a potential marker for apospory in *Pennisetum* (Ozias-Akins *et al.*, 1993). Further analysis using several other RFLP probes mapping to the same area in maize, and additional RAPD and AFLP markers, allowed the construction of a linkage map of the region associated with apomixis in *Brachiaria* (Pessino *et al.*, 1998).

The *Brachiaria* and *Pennisetum* studies used different approaches: in *Brachiaria*, mainly an RFLP maize-based comparative scanning procedure was employed, which yielded a map where only markers located further than 15cM from the apo-locus were found. If the apospory region were hemizygous (as it appears to be the case in

Pennisetum), obviously no marker could have been detected around the apo-locus by using probes isolated from the sexual species. In *Pennisetum* out of 4000 total RAPD markers detected, 12 cosegregated strictly with apomixis, detecting a region that proved to be missing in sexual plants. Ozias-Akins *et al.*, (1998), only detected one marker that partially recombined with apospory. The *Brachiaria* and *Pennisetum* map could be revealing a hemizygous region that is present only in apomicts and confers the capacity for aposporic asexual reproduction. In *Brachiaria* this region is surrounded by probes that map to maize chromosome 5. Unfortunately no comparative studies have been performed to associate both maps because of the lack of common markers with the exception of OPC-04.

b). Identification of molecular marker linked to diplospory

A similar effort is under way with the maize relative *Tripsacum dactyloides*, an apomict that uses diplospory plus pseudogamy. Again, markers were reported in linkage with the region associated with the inheritance of diplospory in this plant (Grimanelli *et al.*, 1998b; Blakey *et al.*, 2001). Suppressed recombination clearly is also a feature of this region, frustrating efforts to clone the critical genes involved in apomixis. Estimates of the size of this region are less accurate than the *Pennisetum*, but it appears to correspond to a 40-cM region of the sexual *Tripsacum* map (Grimanelli *et al.*, 2001a). *Paspalum*, another grass genus, is being also used in a map-based cloning strategy. The apospory plus pseudogamy mechanism of apomixis in this plant is inherited as a simple dominant factor (Martinez *et al.*, 2001). Grasses are known for their high synteny. Pupilli *et al.* (2001) noted the coinheritance of five rice markers with apospory in *Paspulum* and are attempting to clone the locus through their use as probes, again nonrecombination and hemigygosity were detected in association with the locus (Labombarda *et al.*, 2002). Intriguingly, although all of the grasses mentioned above showed considerable synteny with the rice genome, in each case the region associated with the inheritance of apomixis aligns to a different region of that genome (Grimanelli *et al.*, 2001a). Albertini *et al.*, (2001a, 2001b) also reported the isolation of markers linked to apospory in Kentucky bluegrass *(Poa pratensis)*, and Pessino *et al.*, (1997) noted similar findings for *Brachiaria*. Among the eudictoyledonous systems under study, progress is being made towards the mapping of "apomixis" genes in *Taraxacum* (van Dijk *et al.*, 2003;

Vijverberg *et al.*, 2004) and *Erigeron* and for gamete selection in *Taraxacum*, indicating that similar difficulties with cloning also may arise in these plants.

Critically considered, the map-based cloning strategy described above is clearly a valid and promising line of inquiry, but success is certainly not proving to be either easy or assured. The commonly observed suppression of recombination about loci associated with apomixis is particularly problematic, because it markedly reduces the power of the approach. Fine mapping usually is attempted in this method to reduce the distance that must be spanned by a genomic contig, but suppressed crossover frequencies result in a paucity of recombinants and little opportunity to refine the basic mapping data. It also should be remembered that these plants are unlike familiar sexual models such as *Arabidopsis* and rice. They typically have large, polyploid genomes, and almost certainly they have high repetitions of highly repetitive sequences, particularly those of transposons. In most cases, mapping is proceeding in a triploid or tetrapliod highly heterozygous background. Fortuitously, the alleles associated with apomixes typically are simplex and dominant, simplifying phenotypic analysis of segregating progeny. However, once a dominant allele is mapped, contigs must be formed, taking care to ensure that the aligned genomic fragments are from the same homolog. At this time, the hemizygosity seen in *Pennisetum* and *Paspalum* may be an advantage, but it is probably also associated with suppression of recombination mentioned above. Distance of 50 to 100 Mbp appear to be commonly involved in these mapping efforts. Matzk *et al.*, (2003) have suggested that large, hemizygous arrays of DNA linked to apomixis may be attributable to the segregation of markers with a retrotransposon (s) accumulated after the establishment of apomixis. The presence of highly repetitive sequence clustered about the locus of interest will further frustrate efforts in walking and cloning. Additionally, so little is known of the molecular basis of apomixis that the essential elements may not even be recognized even if they were sequenced. Given the regulatory nature of the trait, cis-acting factors, including chromatin-modeling factors, are likely to be as critical as structural sequences (Koltunow and Grossniklaus, 2003a, 2003b). Finally, many of these plants cannot be transformed genetically, so functionally testing of putative control elements is not yet possible. Efforts to address this limitation apparently are under way (Dresselhaus *et al.*, 2001).

Fingerprinting studies

Since apomixis leads to the generation of maternal offspring that are genetically exact copies of the mother plant, segregation analysis of obligate apomicts is untenable. On the other hand, facultative apomictic progenies segregate into apomictic (maternal) and non-apomictic (aberrant) classes (Nogler, 1984a). The individual degree of apomixis is under strict genetic control and remains constant under unperturbed environmental circumstances (Nogler, 1984a). Its evaluation is of principal significance in basic research and the breeding of apomicts. Cytological and embryological analysis of the mother plant and screening of morphologically aberrant progenies are time-consuming and limited methods were traditionally employed for assessing the mode of reproduction. In the last few years, molecular markers have been used in several works for the identification of non-maternal offspring when individual DNA fingerprints revealed a deviation from the maternal profile. Genetic fingerprints have also been successfully used in the identification of hybrids in research programs involving apomicts.

Among the most relevant uses of fingerprinting techniques for the identification of aberrant progeny in apomicts, the following can be quoted as exemplifying the various approaches. RAPD markers have been combined with flow cytometry to determine the genetic origin of aberrant plants within progenies of *Poa pratensis* (Huff and Bara, 1993). Mazzucato *et al.*, (1995) established the degree of apomixis of mother plants in the same species *(Poa pratensis)* by using RAPD and isozymes. Progenies showing different degrees of apomixis were evaluated and morphological analyses were performed at an advanced vegetative state. Hybridization of DNA samples to minisatellite DNA probes has been used in *Rubus* (Antonius and Nybom, 1995) to discriminate between sexual and apomictc progeny. Ortiz *et al.*, (1997) used RAPD and RFLP to determine the degree of apomixis of the mother plants in several accessions of the subtropical forage grass *Paspalum notatum.* Molecular evidences generated in these studies indicated that the combined information of at least three isozymes, three RFLP clones or four to six RAPD primers (depending on the accession) is necessary to accurately ascertain the degree of apomixis of a given genotype.

Molecular studies on apomicts have until now been restricted to the use of isozymes, minisatillites, RFLP and RAPD techniques. The use of SSR (simple sequence

repeat) technology in future studies can improve the reliability, reproducibility, discrimination, standardization and cost effectiveness over the aforementioned method (Smith *et al.*, 1997).

Identification and cloning of transcripts related to apomixis

Differentially expressed genes have been isolated from a wide range of experimental systems by using the differential display techniques (Liang and Pardee, 1992). Molecular techniques based on the PCR (polymerase chain reaction) requireed minute amounts of tissue, and therefore allowed a comparison of gene expression patterns among tissues when sample size was limiting. The isolation of differentially expressed gene by the use of this method could help in the elucidation of the molecular nature of apomixis.

Most of the experimental systems previously used in differential display approaches have taken advantage of the existence of genetically close samples, pairs of mammalian cell populations, isogeneic lines or single genotypes. There is no apomictic system with such a degree of genetic identity, since most apomicts are highly heterozygous and polyploids. Moreover, apomictic reproduction makes it difficult to produce near-isogenic material and non-destructive methods are not available to distinguish ovaries carrying reduced or unreduced embryo sacs from the same plant. Despite these limitations differential display technique has been successfully applied to the identification of transcripts related to the apomictic and sexual pathways by different groups viz. *Pennisetum* (Vielle-Calzada *et al.*, 1996b); *Brachiaria* (Leblanc *et al.*, 1997); *Panicum* (Chen *et al.*, 1999) *and Paspalum* (Pessino *et al.*, 2001) In all the cases, traditional procedures were modified to improved the detection of specific transcripts or avoid the occurrence of false positives. Vielle-Calzada *et al.*, (1996b) incorporated end labeled poly $(A)^+$ anchored primers, which ensured that PCR products contained the poly $(A)^+$ primers on at least one end and avoids detection of fragments that are only flanked by arbitrary decamers. Leblanc *et al.*, (1997), designed a strategy to circumvent the problem caused by the low degree of genetic relatedness available in apomictic systems. The tactic consisted of pooling mRNA populations from ovaries, which originated from different *Brachiaria* hybrid plants sharing the same mode of reproduction. The procedure homogenized the individual's genetic background, making differences due to the mode of reproduction the only apparent distinction between the two bulked samples. Recently

Julio *et al.*, (2003), reported specificity of 65 cloned fragment, checked by reverse northern blot analysis, showed that 11 clones were differentially expressed, 6 in apomictic ovaries, 2 in sexual and 3 in apomictic and sexual, but at different stages. Of the 6 sequences isolated that were preferentially expressed in the apomictic accession, one sequence was from ovaries at megasporogenesis stage; three were from megagametogenesis stage; two were from both stages. Of the two sequences isolated from the sexual accessions, one showed expression in ovaries at megagametogensis, while the other sequence was shown to be specific to both stages. Three sequences were from megasporogenesis stage in apomicts but were also detected at megagametogenesis in sexual plants.

Clones isolated from differential display approaches can be used to target functions specific to tissues or cells. Molecular analysis in sexual species has also yielded several fragments specifically expressed during female gametophytes development and seed formation that can eventually be used in apomicts (Drews *et al.*, 1998). The isolation of more transcripts can be expected shortly, as studies on molecular reproductive biology are rapidly expanding.

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

The details of material used and methods employed in the present study are described under various categories based on the experiments conducted.

3.1 Plant Material

A single sexual plant (IG-96-443) of *Cenchrus ciliaris* was open pollinated to obtain halfsibs, which showed either obligate or facultative apomict (Table 2). Few facultative apomictic halfsibs were further selfed to get F2 segregants that showed clear segregation for mode of reproduction (Table 3). Since the halfsibs are a product of open pollination, no information regarding paternal parent was available. The plant material used in the present study belongs to three halfsibs i.e. 5, 7 and 11. F2 segregants A1 (54) and S1 (5-11) from 5th halfsib, A2 (7-18), A5 (7-13) and S2 (7-4) from 7th halfsib and F2 segregants A3 (11-19), A4 (11-16), S3 (11-17), S4 (11-39), S5 (11-40), S6 (11-58), S7 (11-31), S8 (11-66) from 11th halfsibs were used for fingerprinting, development of marker and subtractive hybridization.

3.2 Methods

3.2.1: Embryo sac analysis

Mature inflorescences were collected from field-grown plants at the time of stigma exsertion and were fixed in formyl acetic acid solution (FAA, 70% alcohol: acetic acid: formaldehyde, 90:5:5 v/v). Fixed pistils were excised and placed in 70% alcohol in a 15mm x 60mm screw-cap vial. A modification of Young's (1979) dehydration and clearing steps was followed, as follows: 70% ethanol; 90% ethanol; 100% ethanol (three changes); ethanol: methyl salicylate 1:1; ethanol: methyl salicylate 1:3; 100% methyl salicylate (two changes).

In this study each excised pistil was placed in a separate vial, and each step was conducted using, 1ml of liquid for 30 minutes. Solutions were changed with a pasture pipette. Cleared pistils were stored in methyl salicylate in vials. For examination in a phase contrast microscope, cleared pistils were mounted in methyl salicylate under an unsealed cover slip on a microscope slide. Components of embryo sacs were examined and photographed.

3.2.2: SDS-polyacrylamide gel electrophoresis

3.2.2.1: Ovule extraction and sample preparation

Inflorescences were collected at the pre-meiotic stage (stigma 10% exerted) and post-meiotic stage (stigma 100% exerted) and were stored in an ice basket. Pistils were removed with the help of needle and forceps under stereo-zoom microscope and were collected in petridishes. The stigma and style of these pistils were excised and about 1,000 ovaries were collected in 500µl of extraction buffer (50mM TrisHCl, pH6.8, containing 10%glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue) in eppendorf tubes and were stored at 4°C. Ovules were thoroughly crushed using a glass rod and centrifuged at 10,000 rpm for 20 min at 4°C. The samples were boiled at 96°C for 5 minutes and were immediately used for electrophoresis.

3.2.2.2: Gel preparation

One dimensional vertical discontinuous polyacrylamide gel electrophoresis was carried out under denaturing condition (i.e. in the presence of 0.1% SDS) as per Laemmli buffer system (1970). Electrophoresis was carried out in 12% resolving and 4% stacking gel. Glass plates of the protein vertical gel electrophoresis (BIO-RAD) were assembled using 0.75 mm spacer like sandwich. The glass plates were locked into the casting stand. Resolving and separating gels were prepared in a 25 ml flask connected to vacuum pump for degassing.

Resolving gel composition (12%)

1.5M TrisHCl, pH8.8	2.5ml
Distilled water	3.5ml
10%(w/v) SDS	100µl
Acrylamide/Bisacrylamide (30%stock))	4.0ml
10% Ammonium persulfate	50µl
TEMED	5µl
Total	**10ml**

Stacking gel composition (4%)

O.5MTrisHCl, pH 6.8	2.5ml
Distilled water	6.1ml
10% (w/v) SDS	100µl
Acrylamide/Bisacrylamide (30%stock))	1.3ml
10% Ammonium persulfate	50µl
TEMED	10 µl
Total	**10ml**

Resolving gel was poured into the sandwich of glass plates using pasture pipette and allowed the gel for 30 to 60 minutes for polymerization at room temperature. After polymerization the upper layer of the gel was washed with distilled water. The stacking gel solution was poured and a 0.75 mm Teflon comb was inserted into the sandwich and allowed stacking gel solution to polymerize for 30 to 45 minutes at room temperature. When polymerization was completed the comb was carefully remove without tearing the edges of the polyacrylamide wells. Wells were rinsed with 1X SDS/electrophoresis buffer to removed unpolymerized monomers.

3.2.2.3: Gel running

The upper and lower buffer chambers were filled with 1X SDS/electrophoresis buffer (TrisHCl, Glycine and SDS). Protein samples were loaded in the well of gel using a syringe with a flat tipped needle. Voltage was kept at 70V until the dye moved into resolving gel and was increased to 100V for complete run. Once the dye reached bottom of the gel, the slabs were removed and the gel was incubated in staining solution.

3.2.2.4: Staining

Gel was incubated in Coomassie blue staining solution (50% methanol, 10% glacial acetic acid, 0.25% Coomassie blue R-250) at room temperature with gentle shaking for at least 30 minutes. The staining solution was discarded and the gel was destained in destaining solution (50% methanol, 10% glacial acetic acid), until the background got cleared.

3.2.3: Isozyme analysis (Native-PAGE)

3.2.3.1: Polyacrylamide gel electrophoresis

Inflorescences were collected at the pre-meiotic and post-meiotic stages and were stored in an ice basket. Pistils were removed with the help of needle and forceps under stero-zoom microscope and were collected in petridishes. The stigma and style of these pistils were excised and about 1,000 ovaries were collected in 500µl of extraction buffer (100mM TrisHCl, PH6.8, 10% sucrose, 1%PVP-40,000 and 10mM β-

mercaptoethanol,) (Soltis *et al*, 1989) at 4°C. Ovules were thoroughly crushed in cold extraction buffer in eppendorf tube using a glass rod. These extracts were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was frozen at –20°C until used for electrophoresis. Electrophoresis was carried out in a vertical discontinuous polyacrylamide gel system (resolving 7.5% and stacking 4%) as per Laemmli *(1970)*. All the procedure including gel preparation and gel running was same as SDS-polyacrlamide gel electrophoresis. Isozymes of Esterase, Glucose-6-phosphate dehydrogenase, Acid phosphatase, Phosphoglucomutase, Phosphoglucoisomerase, Aspartate amino transferase and Superoxide dismutase were studied. The gels were stained as described by Soltis and Soltis (1989).

3.2.3.2: Gel preparation

Resolving gel composition (7.5%)

1.5M TrisHCl, pH8.8	2.5ml
Distilled water	4.35ml
Acrylamide/Bisacrylamide (30%stock))	2.5ml
10% Ammonium persulfate	50µl
TEMED	5µl
Total	**10ml**

Stacking gel composition (4%)

O.5MTrisHCl, pH 6.8	2.5ml
Riboflavin	1.25ml
Distilled water	4.75ml
Acrylamide/Bisacrylamide (30%stock))	1.5ml
10% Ammonium persulfate	50µl
TEMED	10 µl
Total	**10ml**

3.2.3.3: Gel Staining

3.2.3.3.1: Esterase (EST)

Gel was incubated in a solution containing 60mg of α-naphthyl acetate in 5ml of 60% acetone, 20ml of sodium dihydrogen phosphate (0.2M), 10ml of disodium hydrogen phosphate (0.2M), 20ml of distilled water and 80mg of Fast Blue RR salt in 3ml of acetone (about 15minutes in dark at room temperature). After staining the gels were destained in 10% glycerol.

3.2.3.3.2: Acid Phosphatase (ACP)

Gel was stained in a solution containing 1M sodium acetate, pH 5.5, 100mg α-napthyl acetate dissolved in 5ml of 70% acetone and 100mg of Fast Blue RR salt dissolved in 1ml of 10% $MgCl_2$.

3.2.3.3.3: Aspartate Amino Transferase (AAT)

Gel was stained in a solution containing fast blue BB salt (100mg) in 0.1M TrisHCl, pH8.0 with 530 mg L-aspartic acid and 70mg alpha ketoglutaric acid. After 4 hours of incubation, dark blue coloured bands appeared on the gel. Gel was destained in 10% glycerol.

3.2.3.3.4: Phosphoglucomutase (PGM)

Gels was stained in a solution containing 10mg NADP, 15mg MTT, 1mg PMS, 15mg ATP, 175mg $MgCl_2$ in 10ml of 0.1MTrisHCl, pH7.5 with 80mg glucose-1 phosphate and 20 units glucose-6phosphatedehydrogenase.

3.2.3.3.5: Glucose-6-phosphate dehydrogenase (G6PDH)

Gel was stained in a solution containing 5mg NAD, 10mg MTT, 2mg PMS and 50mg $MgCl_2$ in buffer 0.05M TrisHCl, pH8.0 containing 50mg of glucose-6 phosphate disodium salt.

3.2.3.3.6: Superoxide dismutase (SOD)

Gel was incubated in a solution containing 3mg riboflavin, 1mg EDTA and 10mM MTT in 50mM TrisHcl, pH8.0 (50ml). The gel was kept for 20 minutes in dark, then in strong light for 15minutes.

3.2.4: DNA Fingerprinting and development of SCAR marker

3.2.4.1: Isolation of total cellular DNA

Genomic DNA was isolated from the halfsib parent and their F2 segregating population of *Cenchrus ciliaris*, according to Dellaporta *et al* (1983) with a slight modification using CTAB (Cetyl trimethyl ammonium bromide) method. Two grams of field grown healthy leaves were plucked, washed thoroughly and dried. The midrib was removed and the leaves were homogenized to a fine powder using liquid nitrogen, before adding 10ml of lysis buffer (freshly prepared 100mM TrisHCl, (pH8.0), 20mM EDTA, (pH8.0), 1.4M NaCl, 2% CTAB and 1% (w/v) polyvinylpyrrolidine-40,000, 1.5% β-mercaptoethanol). The homogenate was mixed thoroughly by inverting the tube several times and incubated in a water bath at 65°C for 1hr. Equal volume of chloroform:Isoamylalcohol (24:1) was added and mixed thoroughly by gently inverting several times. After centrifugation at 12,000 rpm for 10min at room temperature, the upper aqueous layer was collected, followed by the addition of 1/10 volume of CTAB/NaCl solution (0.7M NaCl and 10% CTAB). An equal volume of chloroform: Isoamylalcohol (24:1) was added, mixed by inverting and centrifuged at 5,000 rpm for 5 minutes at 4°C, took out upper aqueous layer, measured the volume and added same amount of CTAB/ppt solution (50mM TrisHCl, pH 8.0, 10mM EDTA pH8.0 and 1% CTAB). DNA precipitate was cleared, centrifuged at 4,000 rpm at 4°C for 5 minutes and the supernatant was removed and dissolved the pellet in high salt TE buffer (10mM TrisHCl, pH8.0, 0.1mM EDTA, pH8.0 and 1M NaCl). DNA was precipitated by adding 0.6 volume isopropanol. DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The pellet was washed with 70% ethanol, air-dried and dissolved in TE (10mM TrisHCl, pH8.0, 1mM EDTA, pH8.0). RNA was removed by adding RNase @ 10µg/gm tissue, incubated the tubes at 37°C for 1hr and the residual proteins were removed by

extracting once with Phenol: Chloform:Isoamyl alcohol (25:24:1) and twice with Chloform:Isoamyl alcohol (24:1). DNA was precipitated by adding 1/10th volume 3M Sodium acetate, (pH5.2) and 2 volume of ethanol and kept at –20°C overnight. DNA was pelleted at 10,000 rpm, washed with 70% ethanol, air dried and dissolved in TE.

3.2.4.2: DNA amplification by PCR

To identify RAPD fragment linked to apomixis, Bulked Segregant Analysis (BSA) was used (Michelmore *et al,* 1991). Two bulks were prepared by pooling the DNA samples of apomictic (5 apomicts) and sexual (8 sexual) F2 segregants. PCR amplification was carried out in 25 µl of reaction mixture consisting of 10mM TrisHCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 10pM of random decamer primers (Operon Technologies, USA), and 1U of Taq polymerase (Genei, Bangalore) and 10 ng of total genomic DNA. PCR was performed in a PTC 200 thermal cycler (MJ Research, UK). The PCR conditions were as described by Michelmore *et al,* 1991, with slight modifications of 94°C for 4 min, followed by 40 cycles of DNA amplification (45 s at 94°C, 1 min at 37°C and 1min 30 sec at 72°C) and 7 min incubation at 72°C.

Reaction mixture comprised of the following

Cellular DNA	2µl (10ng)
Random primer	1µl (10pM)
dNTP mix (10mM,each)	0.5µl (0.2mM,each)
Taq DNA polymerase (3U/ µl)	0.3µl (1U)
10X Taq polymerase buffer	2.5µl
Nuclease free water	18.7µl
Total	**25µl**

PCR programme followed was

Step 1	Denaturation (94°C)	4 min
Step 2	Denaturation (94°C)	45 sec
Step 3	Annealing (37°C)	1 min
Step 4	Elongation (72°C)	1min 30 sec
Step 5	Follow Step (2-5)	39 times
Step 6	Final Extension (72°C)	7 min
Step 7	Hold (4°C)	6 h
Step 8	End	

The reaction was carried out in a 0.5ml PCR tube and PCR products were run on a 1.5% (w/v) agarose gel with λ-EcoRI/HindIII (MBI Fermentas) DNA marker, and stained in distilled water containing 0.5 μg/ml ethidium bromide for 20 to 30 min and destained in distilled water for 20 to 30 min. DNA bulks were screened using 208 random decamer from A to L series of operon (Operon Tech, USA) primers for identifying RAPD polymorphism. Only bright reproducible RAPD bands were chosen for the study.

3.2.4.3: Cloning and sequencing of PCR products

To convert the selected RAPD bands to SCAR markers, the bands were excised, cloned, and sequenced.

3.2.4.3.1: Isolation of DNA fragment from 1% agarose gel as per gel extraction kit QiagenII (Qiagen, USA)

Polymorphic DNA fragment was excised from the agarose gel with a clean, sharp scalped scissor. Excised gel portion was weighed with eppendorf tube, about 3 volume of Buffer QG was added to 1 volume of gel, incubated the tube at 50°C for 10 minute, mixed the content by vortexing the tube every 2-3 minute during the incubation. When the gel slice dissolved completely, 1 volume of isopropanol was added to the sample and mixed by inverting the tube several times. Minielute column was placed in a 2ml collection tube on a rack, applied the sample to the minielute column, and centrifuged for

1minute, discarded the supernatant and placed the minielute column back into same collection tube. About 500µl of Buffer QG was added to the spin column and centrifuged for 1minute, discarded the supernatant and place the minielute column back in the same collection tube. About 750µl of Buffer PE was added to the minielute column and centrifuged for 1min, discarded the supernatant and centrifuged the minielute column for an additional 1minute at 12,000 rpm. Minielute column was placed into a clean 1.5ml microcentrifuge tube and eluted the DNA in 35µl of elutation buffer. (This kit works on the principle that at ~7.5 pH, DNA adsorbs very tightly to silica gel and elution is performed at pH 7 to 8. Buffer PB is a gel melting solution also containing a pH indicator). Buffer PE is wash solution comprising 70% ethanol.

3.2.4.3.2: Cloning into pGEMT Easy vector (Promega)

The most commonly used thermostable Taq polymerases except Pfu and Vent polymerase often add a single deoxyadenosine in a template independent fashion to the 3 ends of amplified fragments (Clark, 1988) thus providing a compatible overhang. This fact has been used to construct a series of vectors called pGEMT-Easy (Promega), which have 3-T overhangs. The PCR products are easily ligated into this vector and also prevented recircularization of the vector, thereby increasing the efficiency of ligation. Also this vector allows for α-complementation, permitting selection by appearance of blue/white colonies. Also, the multiple cloning sites, flanked by EcoRI sites, which facilitated easy release of insert.

3.2.4.3.3: Ligation of DNA

The vector and insert were taken in the ratio of 1:1,1:3 or 1:5 using the formula

ng of vector X size of insert in Kb X molar ratio of insert: vector / size of vector in Kb=ng of vector

The following reaction was performed in a sterile microcentrifuge tube

Vector DNA	1µl (50-100ng)
Insert DNA	3µl (100ng-1µg)
2X ligase buffer	5µl
T4 DNA ligase (3U/µl)	1µl
Nuclease free water	-
Final volume	**10µl**

The reaction was incubated at 4°C overnight.

3.2.4.3.4: Preparation of Competent Cells

Competent cells were prepared as described in Current Protocols in Molecular Biology (Ausbel *et al,* 1994). The *E.coli* strain DH5α were used for transformation. A single colony of *E.coli* DH5α strain was inoculated into a 5ml LB starter culture and incubated at 37°C overnight. One ml of starter culture was used to inoculate 100ml LB in a 1litre sterile flask and allowed to grow at 37°C with constant shaking (200 rpm) till an OD600 of 0.2 to 0.4 was obtained. The cells were chilled for 30 minutes prior to centrifugation at 4,000 rpm at 4°C for 10 minutes. The bacterial pellet was resuspended in 10 ml of ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM PIPES, pH7.0) and centrifuged at 3,000 rpm at 4°C. The pellet was resuspended again in $CaCl_2$ solution and kept on ice for 30 minutes, centrifuged at 3,000 rpm at 4°C and resuspended finally in 2ml prechilled $CaCl_2$ solution. The cells were then dispensed into aliquots of 100µl in prechilled sterile microcentriifuge tubes. The tubes were kept in ice for 12 to 18 hrs, before transferring to -70°C.

3.2.4.3.5: Transformation of Competent Cells

The appropriate ligation mixture (around 10-50ng DNA) was added to eppendorf tubes containing just thawed competent cells. The tubes were incubated on ice for 30 minutes placed at 42°C for 90 seconds for heat shock and then transferred to ice for 3 to 5 min. 0.75ml of LB was added to the tubes and incubated at 37°C for 1hr with gentle

shaking. Appropriate quantity, i.e. 100µl, 200µl, 400µl of this culture was placed on LA/ampicillin (100mg/ml) plates, which were then incubated at 37°C for 12 to 16 hrs.

3.2.4.3.6: Selection of Transformants

For selection, blue/white colonies appeared after growing them on media containing X-gal (20mg/ml) and 5µl of IPTG (200mg/ml) plates on an LA-ampicillin plate prior to plating of bacterial cultures. The transformed cells developed white colour as a result of non α-complementation (i.e. no β-galactosidase was produced) due to the presence of insert within the multiple cloning site of the vector.

3.2.4.3.7: Plasmid DNA isolation

All clones were maintained in *Escherichia coli* strain DH5α. These included the strains harbouring the genes used as probes for Southern and northern hybridizations and also the constructs generated during the course of investigations. Plasmid DNA was isolated as per the Miniprep kit obtained from Qiagen, USA, which is a modified method based on alkaline lysis method of Binboin and Doly (1979). 5ml of Luria broth (LB) was inoculated with a single bacterial colony on an appropriate antibiotic and incubated overnight at 37°C with constant shaking at 200 rpm. The culture was pelleted by centrifugation at 6,000 rpm for 5minutes. The bacterial pellet was resuspended in 0.3ml of prechilled buffer P1 (50mM TrisHcl, pH8.0, 10mM EDTA, 100µg/ml RNaseA) and vortexed to ensure complete resuspension. 0.3ml of buffer P2 (200mM NaOH, 1% SDS) was added to it, mixed quickly by inverting several times and incubated at room temperature for 5 minutes. 0.3ml of prechilled buffer P3 (3.0M Potassium acetate, pH 5.5) was rapidly but gently added to it, and kept on ice for 5 minutes and then centrifuged at 12,000 rpm for 10 minutes. In the mean time, the Qiagen columns (Anion-exchange resin) were equilibriated with 1ml of equilibriation buffer (750 mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100) and the supernatant obtained from the above step was loaded on the column. The column was then washed twice with wash buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol) to remove the contaminating RNA and protein. The plasmid DNA was eluted by adding 0.8ml of high salt elution buffer (1.25M NaCl, 50mM TrisHcl, pH 8.5, 15% isopropanol) and collected into 2ml microcentifuge tube. To precipitate DNA, 0.7 volume of isopropanol was

added, spun immediately at 10,000 rpm for 20 minutes, washed with 70% ethanol and air-dried. The DNA was then dissolved in appropriate amount of TE.

3.2.4.3.8: Sequencing

Sequencing was carried out by automated sequencing using ABI-prism 310, Genetic analyzer. The sequences obtained were aligned by CLUSTAL W method by using DNASTAR software version 4.05 program. Further, analysis (BLAST, sequence alignment) of the sequences was done using NCBI softwares.

3.2.4.4: Development of SCAR marker

Primers were designed based on the sequence information obtained as above, to amplify the 600bp amplicon unique to apomictic plants. The sequence (5'-3') of the primers were:

Forward: 5'- CAATGTCCAGGACTCCTTTTTGG-3' (23 bp)

Reverse: 5'- AGGTGATAGGGGAATTGCTAAAGT-3' (24 bp)

3.2.4.5: PCR amplification of total genomic DNA of F2 individuals by SCAR primers

PCR was performed using total genomic DNA of F2 segregants as template. The PCR conditions were: 94°C for 4 min followed by 35 cycles of DNA amplification (94°C, 45 s; 63°C, 1min; 72°C, 90 s) and a final incubation at 72°C for 10min. The PCR products were electrophoresed on the 1.2% agarose at a constant voltage of 2V/cm for 3 h. The amplicons were visualized under UV after staining with ethidium bromide.

The reaction was setup as follows

Cellular DNA	1µl (10ng)
Forward primer	1µl (10pM)
Reverse primer	1µl (10pM)
dNTP mix (10mM,each)	0.5µl (0.2mM,each)
10X Taq Buffer containing $MgCl_2$	2.5µl
Taq DNA Polymerase (3U/ µl)	0.3µl (1U)
Nuclease free water	18.7µl
Total	**25µl**

No template DNA was added in the negative control. The reaction was set up in 0.5ml thin walled PCR tube (Axygen) in a MJ Research Thermal Cycler (PTC-200).

The following PCR programme was setup:

Step 1	Denaturation	4min (94°C)
Step 2	Denaturation	45 sec (94°C)
Step 3	Annealing	1min (63°C)
Step 4	Extension	1 min 30 sec (72°C)
Step 5	Repeat steps 2-4	34 times
Step 6	Extension	7 min (72°C)
Step 7	Hold	indefinite (4°C)

3.2.4.6: Southern transfer and hybridization

Two southern transfers were performed, in the first instance the PCR product (RAPD product from OPF-08 primer) was electrophoresed and transferred to the membrane and in second, the total genomic DNA was digested with DraI, electophoresed and transferred on the membrane, both membranes were probed with 600bp eluted polymorphic fragment.

3.2.4.6.1. Southern hybridization (Southern, *1975*)

After the run was completed, the gels were soaked in 0.25N HCl for 15-20 minutes with gentle shaking for depurination. The gel was then washed thrice with double distilled water and soaked in 0.4N NaOH for 45 minutes to 1hr. The assembly for capillary blotting was set up. The gel was carefully kept inverted on a clean glass plate. Nylon N^+ membrane cut to the same size as the gel was placed over it after soaking in 0.4N NaOH for 30 seconds. Then three to five pieces of whatman No. 3 were cut to same size as of the gel, soaked in 0.4N NaOH and placed on the membrane. A clean glass pipette was rolled over to remove the excess NaOH and any air bubbles, which may interfere with the transfer process. 5-7 cm high stack of blotting sheets (of same size as the gel) were placed over the gel followed by a glass plate and a weight of 500-700gms. The transfer was allowed to continue for 14-16 hrs, after which the assembly was dismantled. The membrane was marked with a pencil to indicate the DNA side, soaked in 2X SSC for 1minute, air dried and kept in desiccators till further use.

3.2.4.6.2: Prehybridization and Hybridization

The membrane was placed in a clean, dry hybridization bottle (Amersham) and 15-20ml of pre-hybridization buffer (5X SSPE, 5X Denhardts solution, 0.5% SDS and 0.5ml of 1mg/ml of denatured sonicated salmon sperm DNA) was added to it. Prehybridization was allowed to continue for 2-4 hrs at 65°C at 10 rpm in a hybridization oven.

3.2.4.6.3: Preparation of probe

Random priming

The random priming kit was obtained from MBI Fermentas was used. The reaction was set up in a microcentrifuge tube as per the manufacturers instructions.

DNA	5µl (100-300 ng)
Random primers (hexanucleotide) in 5X buffer	10µl
Sterile water	25µl
Final volume	**40µl**

The mixture was vortexed thoroughly, centrifuged briefly for 5 second and incubated in a boiling water bath for 5-10 minute and quickly chilled in ice. The following were added to it, and incubated at 37°C for 25 minutes. 4µl of dNTP mix were added, incubated further for 5 minutes and reaction was stopped by adding 1mM EDTA. The Probe DNA was added in the hybridization bottle. Hybridization was allowed for 12 to 16 hours at 65°C at 10 rpm in a hybridization oven (Amersham).

3µl of Mix C (dNTP mix minus dCTP)
1µl of Klenow fragment (3units/µl)
1µl of ^{32}P dCTP
5µl of nuclease free water
Final volume 50µl

3.2.4.6.4: Washing of filters and Autoradiography

For almost all filters, high stringency was employed i.e. low salt buffer and high temperature. Initial wash of 2X SSPE, 0.1%(w/v) SDS of 15-20 minutes was followed by 1X SSPE, 0.1%(w/v) SDS for 10 minutes and subsequent washes with 0.5X SSPE and 0.1X SSPE with 0.1%(w/v) SDS. All the washes were carried out at 65°C. The membrane was then kept on a support (used X-ray film), wrapped in a Saran wrap

without trapping air bubbles and exposed to X-ray film (Hyperfilm, Amersham) in a cassette with an intensifying screen. This operation was done in a dark room under red light and the cassette was stored at –70°C. After appropriate exposure time, the cassette was opened in a dark room and a film was developed in Kodak developer for 5 minutes, fixed in Kodak fixer/replenisher for 5 minutes. The film was then air dried after washing thoroughly under tap water.

3.2.4.7: RAPD data analysis

Amplification products were scored for the presence (1) or absence (0) of the bands and binary matrixes were assembled for the RAPD marker. The binary matrix was subjected to the statistical analyses using NTSYS-PC version 2.02. Jacards similarity coefficient was employed to compute pairwise genetic similarities. The similarity matrix was computed and the dendrogram were constructed by applying Unweighted Pair Group Means Averages (UPGMA) using NTSYS-PC.

3.2.4.8: Polymorphism information content (PIC)

The Polymorphism information content (PIC) value described by Anderson *et al.*, (1993) was calculated as follows-

$$PIC = 1 - {}^{n}\sum_{j=1} P_{ij}{}^{2}$$

where, P_{ij} is the frequency of the J^{th} allele for i^{th} marker and summation extends over n alleles.

3.2.4.9: Population genetic analysis

A 36 character amplified fragment data generated from 6 primers and scored from 5 apomicts, one sexual mother and 8 sexual plants was subjected to POPGENE software (version 1.31), for the calculation of various parameters such as gene frequency, allelic richness, effective number of alleles, gene diversity and percent polymorphism.

3.2.5: Subtractive hybridization

PCR based subtractive hybridization method (Reddy *et al.*, 2002) was followed with slight modifications. Subtractive hybridization is a powerful technique for isolating genes expressed or present in one cell population but not in another. It uses a process called driver excess hybridization. Nucleic acid, from which, one wants to isolate differentially expressed sequences (the tester), is hybridized to complementary nucleic acid that is belived to lack the sequence of interest (the driver). Driver nucleic acid is present at much higher concentration (at least 10-fold) than the tester. The driver (sexual) and tester (apomictic) nucleic acid populations were allowed to hybridize and only sequences common to the population can form hybrids. After hybridization, driver-tester hybrids and unhybridized driver were removed. This was the subtractive step. The sequences of nucleic acid that remains behind were enriched for sequences specific to the tester tissue source and depleted for sequences common to the tester and driver. After subtraction, remaining nucleic acid could be used to prepare a library enriched in tester specific clones (Fig. 1).

3.2.5.1: RNA Isolation

For efficient RNA isolation care was taken to keep all glasswares and plasticwares free of contaminating RNase. For this, all solutions, plasticwares and glasswares were treated with 0.1% diethyl pyrocarbomate (DEPC) and kept at 37°C overnight. These were dried in an oven at 80°C and autoclaved at 120°C for 30 minutes to recover any traces of DEPC. Ovaries were ground in liquid nitrogen and powder was lysed in equal volume of lysis buffer (8M Guanidine hydrochloride, 20mM MES, 20mM EDTA pH 7.0, 0.2% β-mercaptoethanol) and incubated for 5 minutes at room temperature. Sample was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was extracted with equal volume of phenol: Chloroform: isoamyal alcohol (25:24:1). The pH of phenol was <6.8, since RNA extraction requires acidic phenol phase. The mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C. Aqueous phase was taken in a fresh eppendorf tube and RNA was precipitated by addition of 0.2 volume prechilled 1M Acetic acid and 0.7 volumes of chilled absolute ethanol to the supernatant

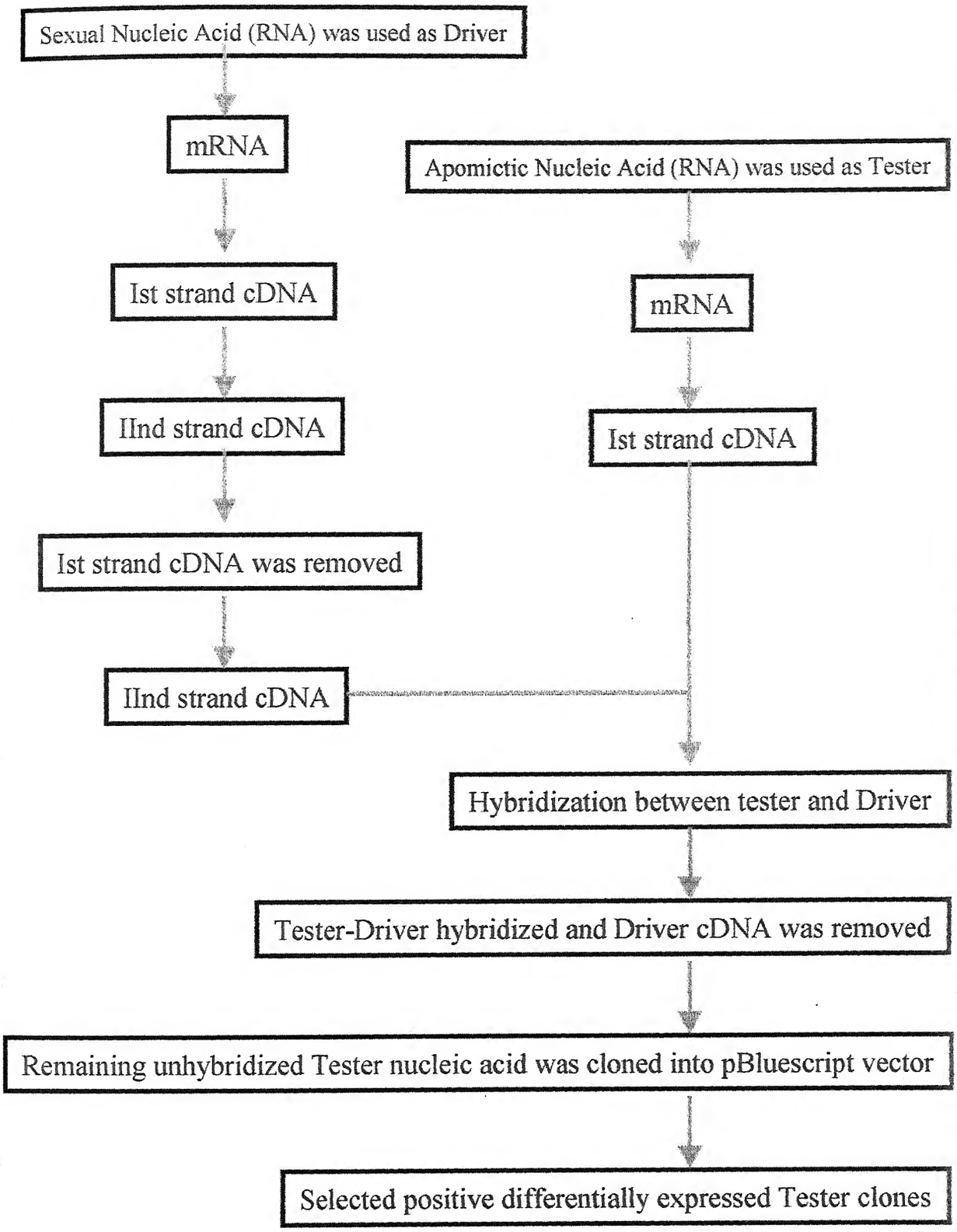


Fig.1: Stages of subtractive hybridization technique used to clone differentially expressed genes in *C. ciliaris*.

and kept at –20°C overnight. RNA pellet was obtained after centrifugation at 10,000 rpm for 10minutes. The pellet was washed in 0.2M Sodium acetate, (pH5.2) followed by 70% ethanol, air-dried and dissolved in DEPC treated water. RNA pellets were dissolved through vortxing or heating at 65°C in a water bath for 10-15minutes.

3.2.5.2: mRNA Isolation

Magnetic beads coupled to oligo (dT) were used for efficient mRNA isolation from total cellular RNA. Here oligo $(dT)_{18}$ mRNA purification system was used, which employs paramagnetic particles linked to an oligo (dT) primer. The steps involved were as follows. The poly$(A)^+$ fraction (mRNA) is hybridized under high salt condition with the oligo(dT) primer, which is coupled to paramagnetic particles. The poly $(A)^+$ fraction (mainly ribosomal RNA) is left unbound. The paramagnetic particles with the attached poly $(A)^+$ RNA are captured using a magnet and washed. The poly $(A)^+$ is eluted from the paramagnetic particles using RNase free water.

3.2.5.2.1: Annealing of probe and washing of particles

Initially about 1mg of RNA was taken into the eppendorf tube and equal volume of 2X-binding buffer (20mMTrisHCl, pH7.5, 1M LiCl, 2mM EDTA) was added and heated at 65°C for 2 minutes and cooled on ice. About double the concentration of RNA i.e. 2mg oligo$(dT)_{18}$ was taken into another eppendorf tube and resuspended by flicking the tube gently. Tube was placed in magnetic rack for 30 sec for the collection of magnetic beads on tube side. Supernatant was discarded. Bead was washed with 200µl of 2X binding buffer and captured with the magnet before discarding supernatant, bead was resuspended in 200µl, 2X-binding buffer.

3.2.5.2.2: Capture and washing of hybrids

Total RNA and magnetic beads were mixed and left at room temperature for 5 minutes. Magnetic beads were captured by magnet, washed the beads with 200µl of 1X washing buffer (10mM TrisHCl, pH7.5, 0.15M LiCl, 1mM EDTA) and captured with the magnet as above, supernatant was discarded, this washing step was repeated and after the final wash and capture. The supernatant was removed as much as possible without disturbing the beads.

3.2.5.2.3: Elution of mRNA

Beads were resuspended by adding the 20µl 1X elution buffer (2mM EDTA), tubes were heated at 65°C for 2 minutes and placed the tube immediately in the magnetic rack. Beads were captured by magnet and supernatant was collected, which contained purified mRNA.

3.2.5.3: First strand cDNA synthesis

In two separate tubes one for tester and another for driver, at least 10µg poly(A)$^+$ RNA was prepared at a concentration of 1µg/µl. For tester, added tester dT and for driver added driver dT in a final concentration of 50µg/µl, mixed by vortexing, briefly centrifuged and added 500µM of each dNTPs, 1X RT buffer, 10mM DTT and finally 20µl (200U) AMV reverse transcriptase for a final concentration of 1000U/ml in total 200µl. Incubated the tube at 37°C for 10min, then placed at 42°C for 1 hr. RNA was removed from RNA-DNA hybrid by RNAsin or RNaseH (50U).

Sequence of Tester dT (1µg/µl)

5'-TATAGATCTGCGGCCGCAAGCTTTTTTTTTTTTTTTT-3'

Sequence of Driver dT (1µg/µl)

5'-GTAATACGACTCACTATAGGGTTTTTTTTTTTTTTTT-3'

(5mM) dNTPs	20µl (500µm final, each)
(5X) RT Buffer	40µl (1X final)
(200mM) DTT	10µl (10mM final)
(0.5mg/ml) oligo (dT) $_{18}$	20µl (50µg/ml final)
Distilled water	80µl
RNAsin (5U/µl)	10µl (50U/µl, final)
AMV reverse transcriptase	20µl (200U)
Total	**200 µl**

3.2.5.4: Purification of the first cDNA strand

When the reaction was completed, about 4µl of 0.5M EDTA pH 8.0 was added to stop the reaction. DNA was purified once with phenol: chloroform: isoamylalcohol (25:24:1) and twice with chloroform: isoamylalcohol (24:1). DNA was precipitated by adding 1/10 of 3M Sodium acetate pH5.2 and 2 volume of absolute alcohol and kept at –20°C for 30 minutes. DNA was pelleted at 10,000 rpm, washed with 70% ethanol, air dried and dissolved in 52µl of DEPC water.

3.2.5.5: Adaptor Ligation

Adaptor was ligated in both the tester and driver first strand cDNAs. In a two separate tubes ligation was setup in 50µl of total ligation mix, added 1X ligase buffer, 20ng of tester adaptor for tester and driver adaptor for driver and 3U of T4 RNA ligase. The ligation mix was incubated at 37°C for 1 hour, and then placed at 25°C for over night. Again purification was done as above and pooled pellet was resuspended in 63µl of 2X PCR buffer.

Sequence of Tester RNA LIG

5'- GCTAGCATATGGGCCCGAATTCC-3'

Sequence of Driver RNA LIG

5'- CCCTTTAGTGAGGGTTAATTTC-3'

In two separate tubes following were added

10X T4 RNA ligase buffer	5µl
Adaptor (150ng/ µl)	1µl
T4 RNA ligase (3U/ µl)	1µl
DNA (first strand)	43µl
Total	**50 µl**

3.2.5.6: Second strand cDNA Synthesis of Driver

PCR was carried out for the second strand cDNA synthesis of driver. The forward primer, which was biotin labeled, and the reverse primer were used in 100µl of total reaction. The reaction and PCR condition was as follows.

10X buffer	6µl
dNTPs (10mM)	16µl
Forward primer i.e. biotin labeled (150ng/µl)	4µl
Reverse primer (150ng/µl)	4µl
Taq polymerase (5U/µl)	4µl
Template i.e. first strand cDNA always in 2X buffer	6µl
DEPC water	60µl
Total	**100µl**

Sequence of Driver forward primer (150ng/µl) (biotin labeled)

5'-GAAATTAACCCTCACTAAAGGG-3'

Sequence of Driver reverse primer (150ng/µl)

5'-GTAATACGACTCACTATAGGGTT-3'

PCR Programme

Step 1	Denaturation	94°C (2min)
Step 2	Denaturation	94°C (30 sec)
Step 3	Annealing	55°C (1min)
Step 4	Extension	72°C (3min)
Step 5	Followed 2-4	29 times
Step 6	Final extension	72°C (7min)
Step 7	Hold	4°C (infinite)

3.2.5.7: Removal of first strand cDNA of Driver

The first and second cDNA strand was separated by (0.15N NaOH) alkaline treatment and the strands were removed by magnetic beads. Second strand cDNA was washed with 2x PCR buffer and eluted in 50µl of 2x PCR buffer.

3.2.5.8: Hybridization of tester first strand and driver second strand

Hybridization was carried out at 65°C for 12 to 16 hours. Hybridization reaction was set up in 0.5ml PCR tube. Incubated the reaction mix (50µl of tester first strand and 50µl of driver second strand) at 65°C in PCR for 12 to 16 hours. Hybridized driver-tester common sequences and unhybridized driver second strand cDNA was removed by magnetic beads, unhybridized tester first strand cDNA was taken out.

3.2.5.9: PCR amplification of the unhybridized tester first strand cDNA

Unhybridized tester first strand cDNA was amplified with forward and reverse primers having ApaI and NotI sites. PCR product was purified as above and eluted in 50µl water.

P1 (forward primer)

5'-GGAATTCGGGCCCCATATGCTAGC-3'

P2 (reverse primer)

5'-TATAGATCTGCGGCCGCAAGCTT-3'

PCR condition was as follows

10X Buffer	3µl
dNTPs mix (10mM)	8µl
Forward primer (150ng/µl)	2µl
Reverse primer (150ng/µl)	2µl
Taq polymerase (5U/µl)	1µl
Template DNA i.e. always in 2X buffer	30 µl
DEPC water	4µl
Total	**50µl**

PCR Programme

Step 1	Denaturation	94°C (2min)
Step 2	Denaturation	94°C (30 sec)
Step 3	Annealing	55°C (1min)
Step 4	Extension	72°C (3min)
Step 5	Followed 2-4	29 times
Step 6	Final extension	72°C (7min)
Step 7	Hold	4°C (infinite)

3.2.5.10: Restriction digestion of PCR product and vector (pBluescript KS +/-)

PCR product and vector were separately digested with ApaI and NotI for efficient cloning of the cDNAs into the vector. Digestion reaction was set up in 1X buffer, 20U enzyme and 2µg of the DNA in a total 60µl of reaction mix.

3.2.5.10.1: ApaI digestion

Reaction mix was incubated at 30°C for 3 to 4 hours, purified as above, by phenol: chloroform, dried and dissolved in 50µl of autoclaved distilled water.

DNA (20ng/µl)	48µl
10X buffer	6 µl
ApaI (10Uµl)	2µl
Distilled water	4µl
Total	**60µl**

3.2.5.10.2: NotI digestion

Reaction mix was incubated at 30°C for 3 to 4 hours, purified as above, by phenol: chloroform, dried and dissolved in 50µl of autoclaved distilled water.

DNA (20ng/µl)	48µl
10X buffer	6µl
NotI (10U/µl)	2µl
Distilled water	4µl
Total	**60µl**

3.2.5.11: Ligaton of Vector and Insert

Set up ligation reaction as follows, incubated the ligation mix at 4°C overnight

Insert DNA	6µl
Vector DNA	1µl
10X Buffer	2µl
T4 DNA ligase (3U/µl)	2µl
Distilled water	9µl
Total	**20µl**

3.2.5.12: Transformation and selection

The appropriate ligation mixture (around 10-50ng DNA) was added to eppendorf tubes containing just thawed competent cells. The tubes were incubated on ice for 30 minutes, placed at 42°C for 90 seconds for heat shock and then transferred on ice for 3 to 5 min. 0.75ml of LB was added to the tubes and incubated at 37°C for 1hr with gentle shaking. Appropriate quantity, i.e. 100µl, 200µl, 400µl of this culture was placed on LA/ampicillin (100mg/ml) plates, which were then incubated at 37°C for 12 to 16 hrs. For selection, blue/white colonies appeared after growing them on media containing X-gal (20mg/ml) and 5µl of IPTG (200mg/ml) plates on an LA-ampicillin plate prior to

plating of bacterial cultures. The transformed cells developed, white colour as a result of non α-complementation (i.e. no β-galactosidase was produced) due to the presence of insert within the multiple cloning site of the vector.

3.2.5.13: Sequencing

Sequencing was carried out by automated sequencing using ABI-prism 310 Genetic analyzer. The sequences obtained were aligned by CLUSTAL W method by using DNASTAR software version 4.05. Further, analysis (BLAST, sequence alignment) of the sequences was done using on NCBI softwares.

3.2.4.14: Bioinformatic analysis

Bioinformatic analysis was carried out in several steps. In the most favorable case, the clones were submitted to the databases of the National Center for Biotechnology Information (NCBI) for Blastx and Blastn. Translation analysis was carried out using the Edit Sequence Programme of DNA STAR 4.05. Amino acid sequence was submitted to various programmes available on NET for analysis.

First of all, the amino acid sequence was submitted to protein colourer tool of EMBL-EBI (http://www.expasy.org/tools/protparam.html) for amino acid color. In order to search for conserved domain, the amino acid sequence was submitted to the NCBI conserved domain finder (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). Using the PROSITE pattern database of expert protein analysis system (http://www.expasy.org/cgi-bin/prosite/scanview.cgi) the sequence was scanned and various sites present in sequence were recorded (Post-translational modification sites). Secondary structure prediction was done by using SOPMA (significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments) software of NPS (network protein analysis). Phylogenetic analysis was carried out using the Megaalign programme of DNASTAR 4.05.

3.2.5.15: Northern Analysis

3.2.5.15.1: Resolution of RNA on agarose-formaldehyde gels

Cellular RNAs are generally present as secondary and tertiary structures, which may interfere with proper separation on agarose gels. To keep them in a single stranded form, formaldehyde @ 0.8-2.2M was used in the agarose gels and also in the loading premix. All the glasswares were sterilized with DEPC. The gel assembly was treated overnight with 0.5% hydrogen peroxide solution and then rinsed thoroughly with DEPC treated water.

3.2.5.15.2: Preparation of gel

A 1.2% agarose gel was prepared by melting 1.2 gm agarose in 92ml of DEPC treated water. It was cooled to 50°C-60°C and 5ml of 20X gel running buffer (800mM MOPS, 200mM Sodium acetate, 40mM EDTA, pH 7.0) and 3ml of formaldehyde were added to it. After mixing the contents thoroughly, it was poured in the gel casting tray. After polymerization the gel was pre run at 40V, for 10-15minutes.

3.2.5.15.3: Preparation of RNA sample

20µg of RNA sample was mixed with 1X running buffer, 50% formamide, and 2.2M formaldehyde (denaturation premix) and incubated at 65°C for 15-20 minutes. To each sample $1/10^{th}$ volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 1mM EDTA, 50% glycerol in DEPC treated water) was added. The samples were loaded on the gel and electrophoresced at 90V for 3 hrs. Since MOPS buffer is light and heat sensitive, electrophoresis was carriedout in dark and the buffer was changed once or twice during the run. After the run, the lane containing the RNA marker was excised out and stained with ethidium bromide and rest of the gel was processed as follows.

3.2.5.15.4: Northern transfer and hybridization

Transfer of RNA from agarose-formaldehyde gels to nylon membrane (+Ve charged) by wet blotting (Sambrook *et al.*, 1982). The gel was placed in a clean, sterile DEPC treated glass tray and rinsed in DEPC treated water for 15 minutes with gentle shaking. This was followed by a 45 minutes wash in 20X SSC at room temperature. For wet blotting a glass plate covered by a wick of Whatman No. 3 paper was dipped in a tank of 20X SSC. The gel was placed upside down on the wick, followed by the membrane (Qiagen N) and 3-5 pieces of presoaked Whatman No. 3 paper of same size. Care was taken to avoid trapping of air bubbles and stack of 5-7cms of blotting sheets was placed on the assembly. This was followed by a glass plate and 500gm weight. The blotting was allowed to continue for 16-18 hrs. The nylon N^+ membrane was then lifted and RNA was immobilized either through baking at 80°C for 2 hrs or by UV cross-linking at 245nm for 1 to 5 minutes. The membrane was dipped in 2X SSC, air dried and stored in a desiccator until further use.

3.2.5.15.5: Prehybrization

This was carried out in prehybridization tube in a hybridization oven (Amershamn). The membrane was placed in prehybridization solution (50% formamide, 5X Denhardts solution, 5X SSPE and 0.1%(w/v) SDS and denaturated sonicated salmon sperm DNA@ 100µg/ml) and incubated at 42°C in a hybridization oven for 6-12 hours. The rest of the procedure, i.e. labeling of probe, hybridization, washing of filter and autoradiography were performed in the same way as described for southern hybridization (in section 3.2.4.6).

3.2.5.16. Characterization of cDNA fragments

To characterize 11 clones, internal primers were designed for each clone using primer design programme of DNASTAR 4.05. Using these primers, the genomic DNA and plasmid DNA containing the insert were amplified. The reaction was setup as follows.

DNA	1µl (10ng)
Forward primer	1µl (10pM)
Reverse primer	1µl (10pM)
dNTP mix (10mM,each)	0.5µl (0.2mM,each)
10X Taq Buffer containing $MgCl_2$	2.5µl
Taq DNA Polymerase (3U/ µl)	0.3µl (1U)
Nuclease free water	18.7µl
Total	**25µl**

No template DNA was added in the negative control tube. The reaction was set up in 0.5ml thin walled PCR tube (Axygen) in a MJ Research Thermal Cycler (PTC-200).

The following PCR programme was followed:

Step 1	Denaturation	94°C (2min)
Step 2	Denaturation	94°C (30 sec)
Step 3	Annealing	55°C (1min)
Step 4	Extension	72°C (1.5min)
Step 5	Followed 2-4	34 times
Step 6	Final extension	72°C (7min)
Step 7	Hold	4°C (infinite)

Procurement of chemicals, kits and plasticware

All restriction enzymes and RNA markers were obtained from Boehringer Mannheim (Roche) pGEMT-Easy vector kit, T4 DNA ligase, dNTP mix, λ-HindIII and λ-HindIII/EcoRI marker and random priming kit were procured from MBI Fermentas. Plasmid DNA mini kit, Qiagen gel extraction kit and Nylon N+ membrane was procured from Amershamn.

Random primers (OPA to OPL series) were obtained from Operon Tech. USA. The primers were procured from Co Genei Banglore, ^{32}P dCTP was procured from BARC, Bombay. Others Chemicasl and solutions were procured from Sigma chemical Co. USA, USB, SRL, Qiagen and Hi-media. Ethanol was procured from Bengal chemical and plasticware from Tarson and Axygen, while glassware was purchased from Schott, Germany and Vensil, Bangalore.

RESULTS

CHAPTER 4
RESULTS

Studies on isolation of genes associated with apomixis were conducted using different methodologies. The results of various experiments conducted are compiled and given below.

4.1: Development of mapping population

The open pollination of obligate sexual plant (IG-96-443) gave rise to 11 half-sibs, out of which six genotypes were found to be obligate apomicts while the rest were facultative ones. The only controlled cross has been found to be an obligate apomict (Table 2). Among the half-sibs, the facultative plants were further selfed to generate F2 plants and this resulted into 3 different classes viz. obligate apomicts, sexual and facultative apomicts in different proportions (Table 3). F2 segregants from four halfsibs did not indicate any genetic pattern of segregation. From these F2 populations, only obligate sexual and obligate apomicts were further chosen for this study.

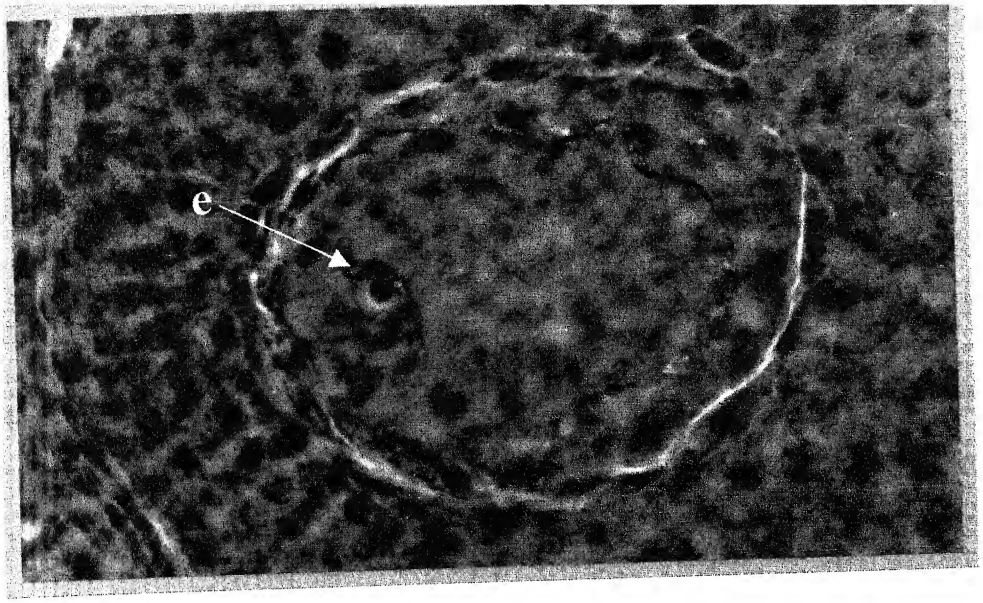
4.2: Embryo sac analysis

Embryo sac analysis was carried out using ovule-clearing technique of Young *et al.*, (1979) in the half-sibs and F2 segregants to classify their mode of reproduction. The Methyl salicylate clearing could help in examining the embryo sacs. There were three different kinds of ovules

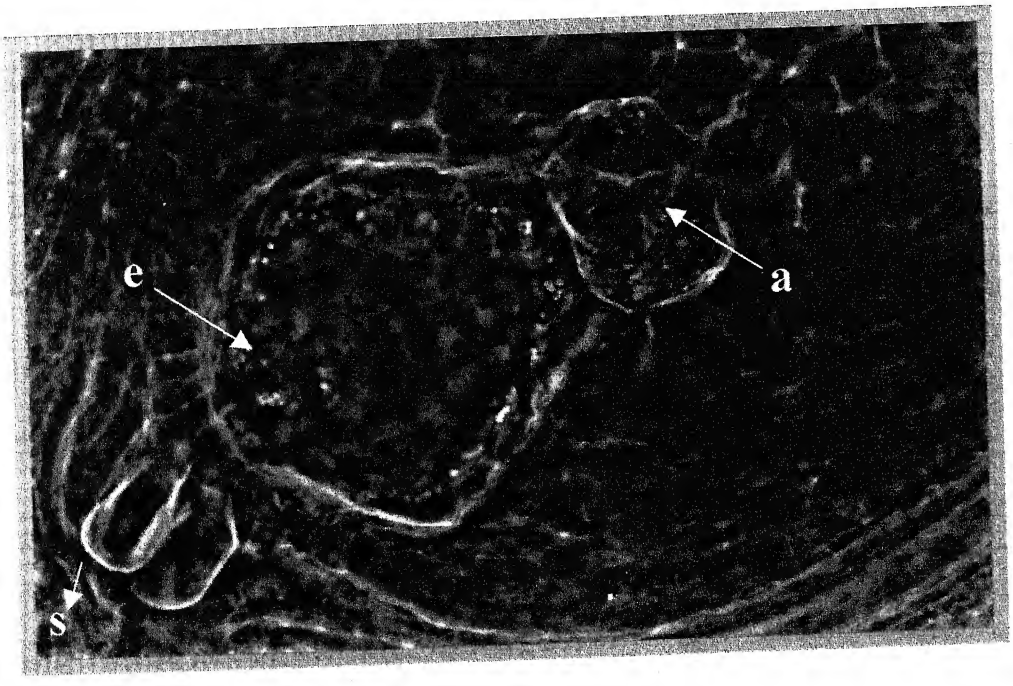
a). Ovules bearing single seven celled meiotic embryo sac characterized by the egg apparatus (egg cell and two synergids), a large two-nucleated central cell and a mass of proliferated antipodals at the chalazal end (Fig. 2a).

b). Ovules bearing one to several aposporous embryo sacs (Fig. 2b). These sacs showed different sizes (30-60μm), constitution, shape and orientation. The largest ones usually contained two large polar nuclei in a widely vacuolated central cell, the egg cell, and one or two synergids. The absence of antipodals characterized the aposporous sacs.

c). Ovules with one meiotic embryo sac surrounded by one to several aposporous sacs.



a: Apomictic embryo sac



b: Sexual embryo sac

Fig. 2: Cleared Embryo Sacs of *Cenchrus ciliaris*

A: Apomictic embryo sac

B: Sexual embryo sac

a: antipodals; e: eggcell; s: synergid

Table 2. Half-sibs and hybrids of *C. ciliaris* with their modes of reproduction determined using ovule clearing technique

Half-sib/Hybrid No.	Type of cross	Mode of reproduction
1	Open pollinated	Facultative apomicitc
2	Open pollinated	Obligate apomicitc
3	Open pollinated	Facultative apomicitc
4	Open pollinated	Obligate apomicitc
5	Open pollinated	Facultative apomicitc
6	Open pollinated	Facultative apomicitc
7	Open pollinated	Facultative apomicitc
8	Open pollinated	Obligate apomicitc
9	Open pollinated	-
10	Open pollinated	Obligate apomicitc
11	Open pollinated	Facultative apomicitc
12	IG-96-443 x IG-96-66	Obligate apomicitc

Table 3. Different modes of reproduction of F2 segregants developed from four facultative apomictic half-sibs

F1 hybrid plant No.	Completely apomictic (F2)	Completely sexual (F2)	Facultative apomictic (F2)
3	11	0	19
5	4	1	14
7	2	3	13
11	3	12	28

4.3: Protein polymorphism

4.3.1: Native and SDS-PAGE analysis

A total of 17 protein bands were observed out of which four proteins exhibited polymorphism among ovaries of sexual and apomictic plants (Table 4). 97KDa protein was associated with only pre meiotic sexual. While 80KDa protein complement was absent only in apomictic post meiotic ovaries. A 60KDa protein was observed in sexual ovaries undergoing both pre meiotic and post meiotic stages, it was conspicuously absent in both the stages of apomictic ovaries. Another 50KDa protein was associated with pre meiotic ovaries of both the sexual and apomictic plants, while it was absent in post meiotic ovules of both the plants (Fig 3). Thus, four different bands of proteins were found with different patterns during pre meiotic and post meiotic megasporogenesis and megagametogenesis. This clearly showd that stage specific proteins are expressed during apomictic and sexual developmental pathways.

4.3.2: Dendrogram analysis

Dendrogram analysis based on protein banding pattern indicated two major groups. One group included pre meiotic apomictic and sexual ovaries, while the post meiotic and sexual ovaries grouped into another (Fig. 4). This is in contrast to dendrogram based on isozyme analysis. Protein band similarity indicated clear distinction between two different stages in both the types of ovaries, which further implied that proteins are more differentiated between pre and post meiotic megasporogenesis and megagametogenesis than among sexual and apomictic types.

4.4: Isozyme polymorphism

4.4.1: Zymogram analysis

Extracts from four different kinds of ovules of obligate apomictic and sexual plants were analyzed for six different enzyme systems. Enzymes exhibited polymorphism among ovules of 4 stages. Out of 43 putative alleles scored among 14 loci, pre meiotic ovules exhibited higher no. of alleles (37), while post meiotic ovules

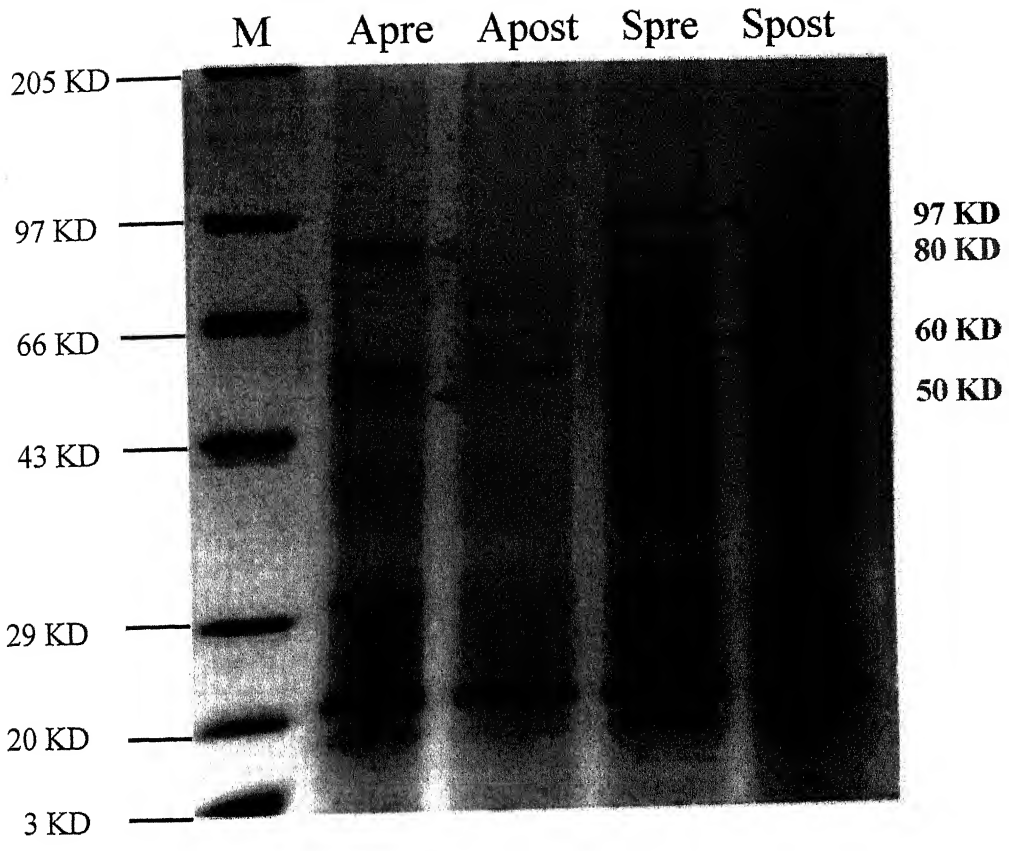


Fig. 3: Protein polymorphism among pre and post meiotic ovaries of apomictic and sexual F2 segregants of *Cenchrus ciliaris* at different stages.

M: Protein molecular weight marker
Apre: Apomictic pre-meiotic stage
Apro: Apomictic post-meiotic stage
Spre: Sexual pre-meiotic stage
Spost: Sexual post-meiotic stage

Table 4. Protein complements of different sizes associated with pre and post meiotic stages of apomictic and sexual F2 segregants

Protein complements	MW (KDa)	Apomitic (7-18)		Sexual (7-4)	
		Pre-meiotic	Post-meiotic	Pre-meiotic	Post-meiotic
1	97	-	-	+	-
2	80	+	-	+	+
3	60	-	-	+	+
4	50	+	-	+	-

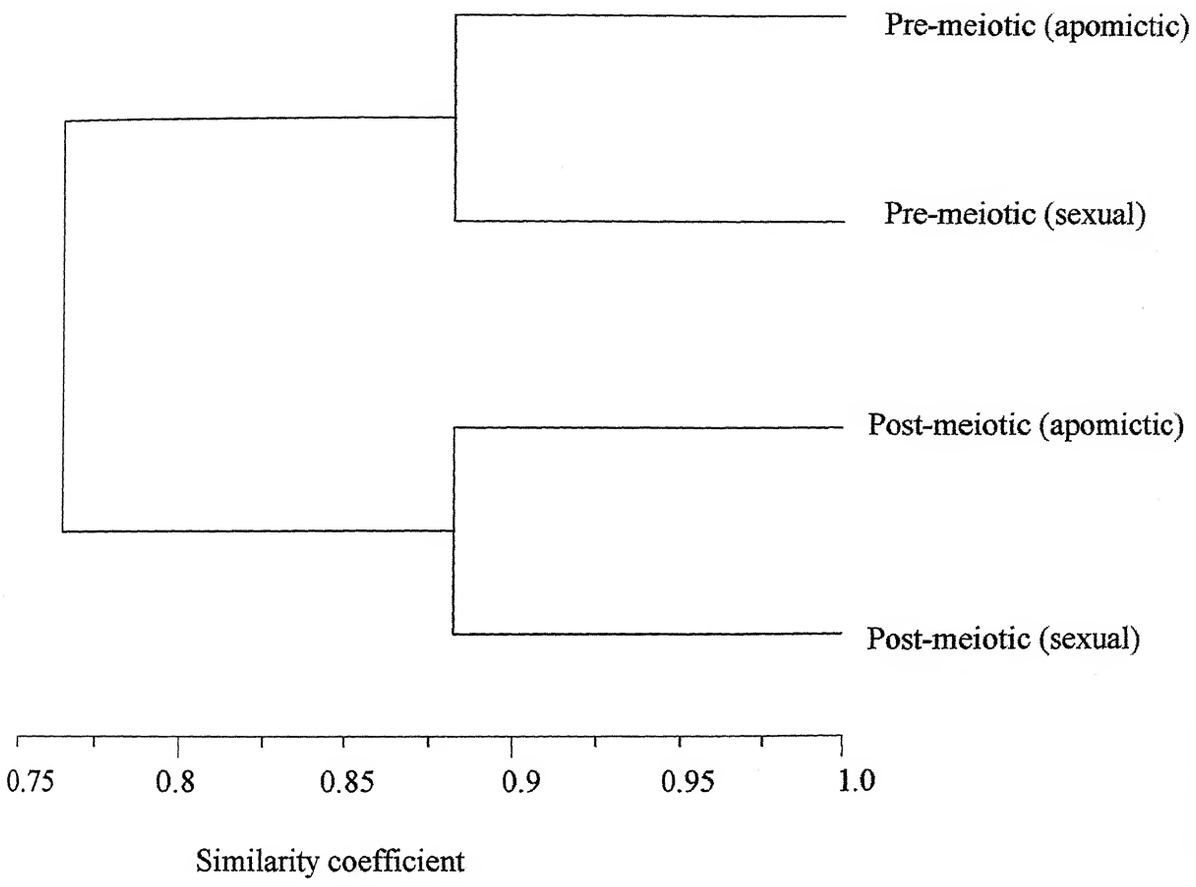


Fig. 4: UPGMA dendrogram depicting relationship among pre and post meiotic ovaries of apomictic and sexual plants based on protein polymorphism analysis

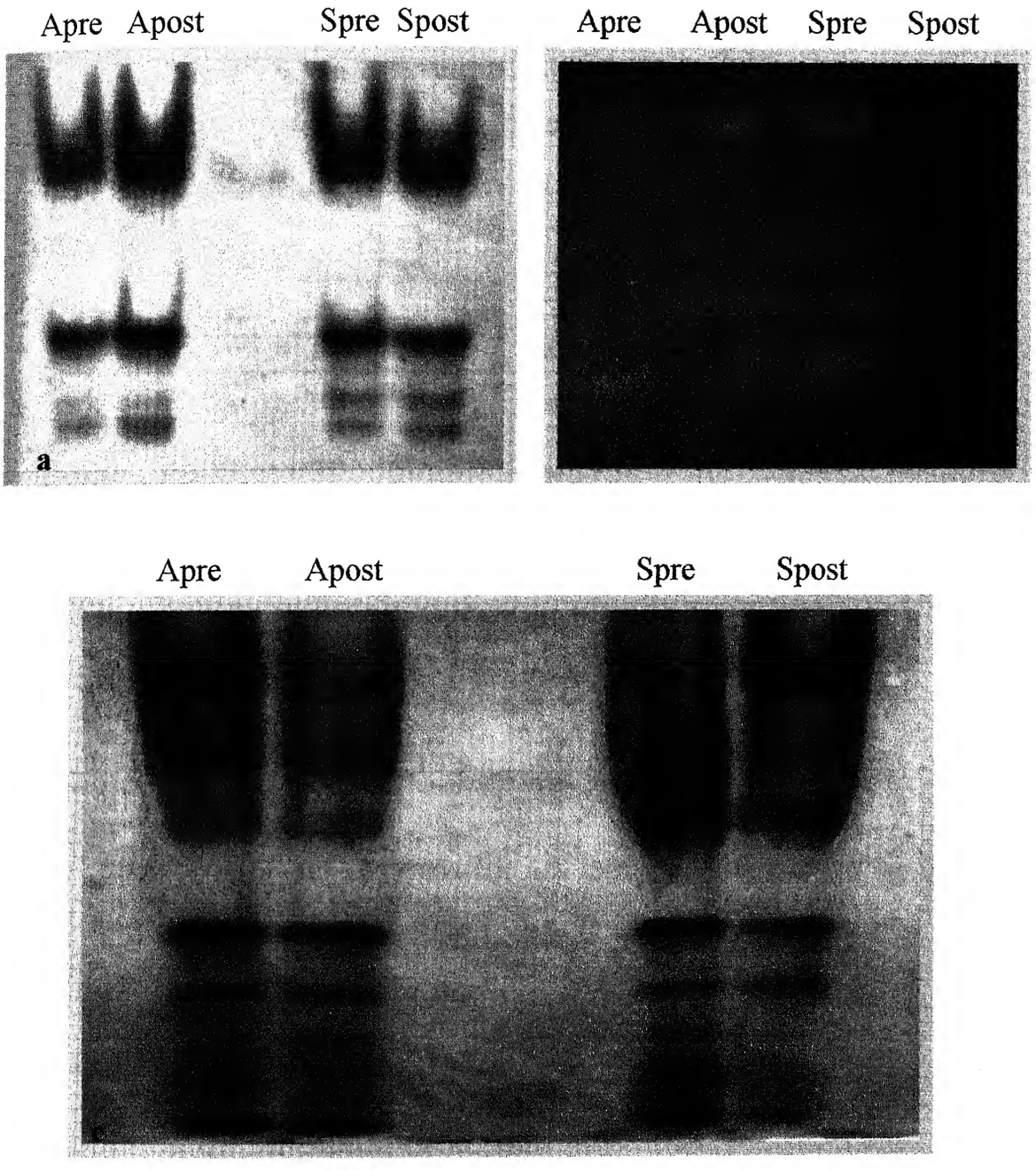


Fig. 5: Isozyme polymorphism among pre and post meiotic ovaries of apomictic (Apre & Apost) and sexual (Spre & Spost) F2 segregants of *Cenchrus ciliaris*

A: Acid amino transferase (AAT)
B: Superoxide dismutase (SOD)
C: Esterase (EST)

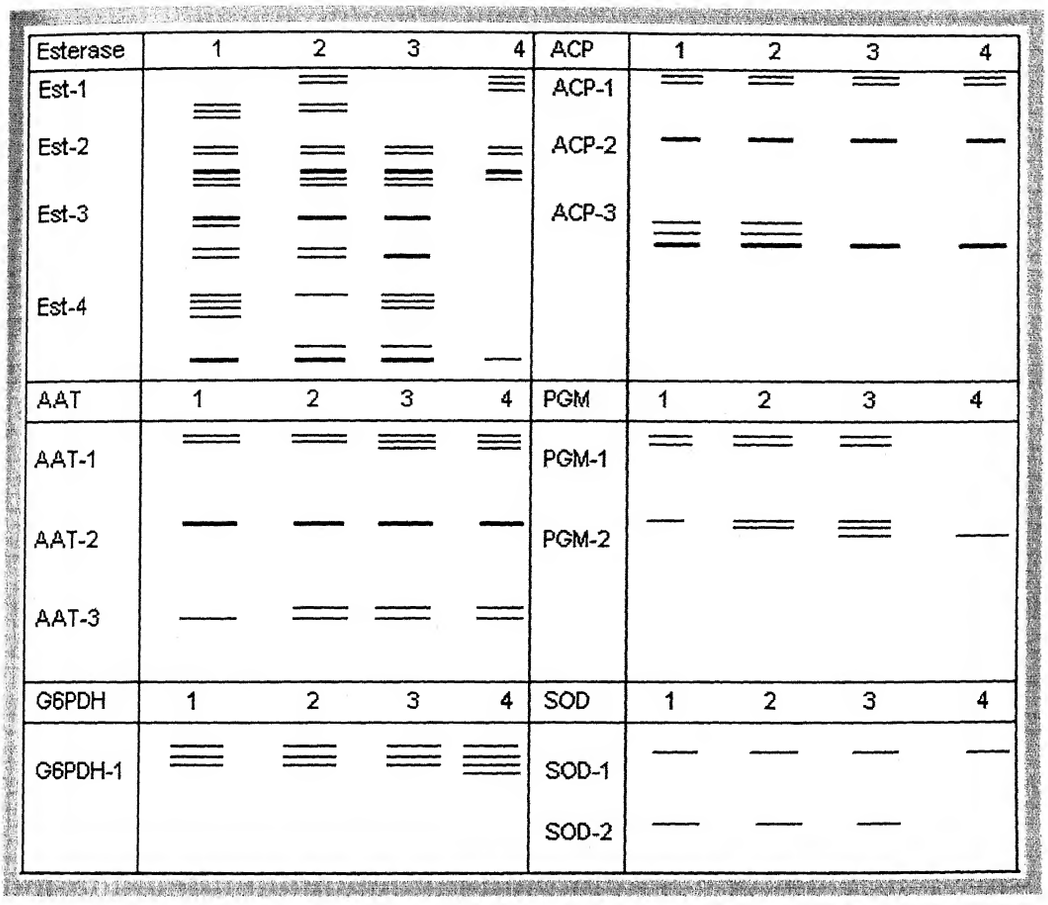


Fig. 6: Zymogram patterns for six isozymes of *Cenchrus ciliaris.*

EST: Esterase
ACP: Acid phosphatase
AAT: Aspartate amino transferase
PGM: Phosphoglucomutase
G6PDH: Glucose-6-phosphate dehydrogenase
SOD: Superoxide dismutase

Lane 1: Apomictic pre-meiotic stage
Lane 2:Apomictic post-meiotic stage
Lane 3:Sexual pre-meiotic stage
Lane 4: Sexual post-meiotic stage

Table 5. Isozyme polymorphism associated with pre and post meiotic stages of apomictic and sexual F2 segregants

S.N.	Enzyme	Code	No.of loci recorded	No. of alleles			
A. Isozymes				Apomictic		Sexual	
				Pre	Post	Pre	Post
1.	Esterase	E.C.3.1.1	4	20	15	12	8
2.	Acid phosphatase	E.C.3.1.1.2	3	6	6	4	4
3.	Aspartate aminotransferase	E.C.2.6.1.1	3	4	5	6	6
4.	Phosphoglucomutase	E.C.5.4.2.2	2	2	4	5	1
5.	Glucose6phosphodehydrogenase	E.C.1.1.1.4.2	1	3	3	3	4
6.	Superoxide dismutase	E.C.1.15.1.1	1	2	2	2	1
Total			14	37	35	32	24
B. Protein Complements				15	12	17	14
Grand Total				52	27	49	38

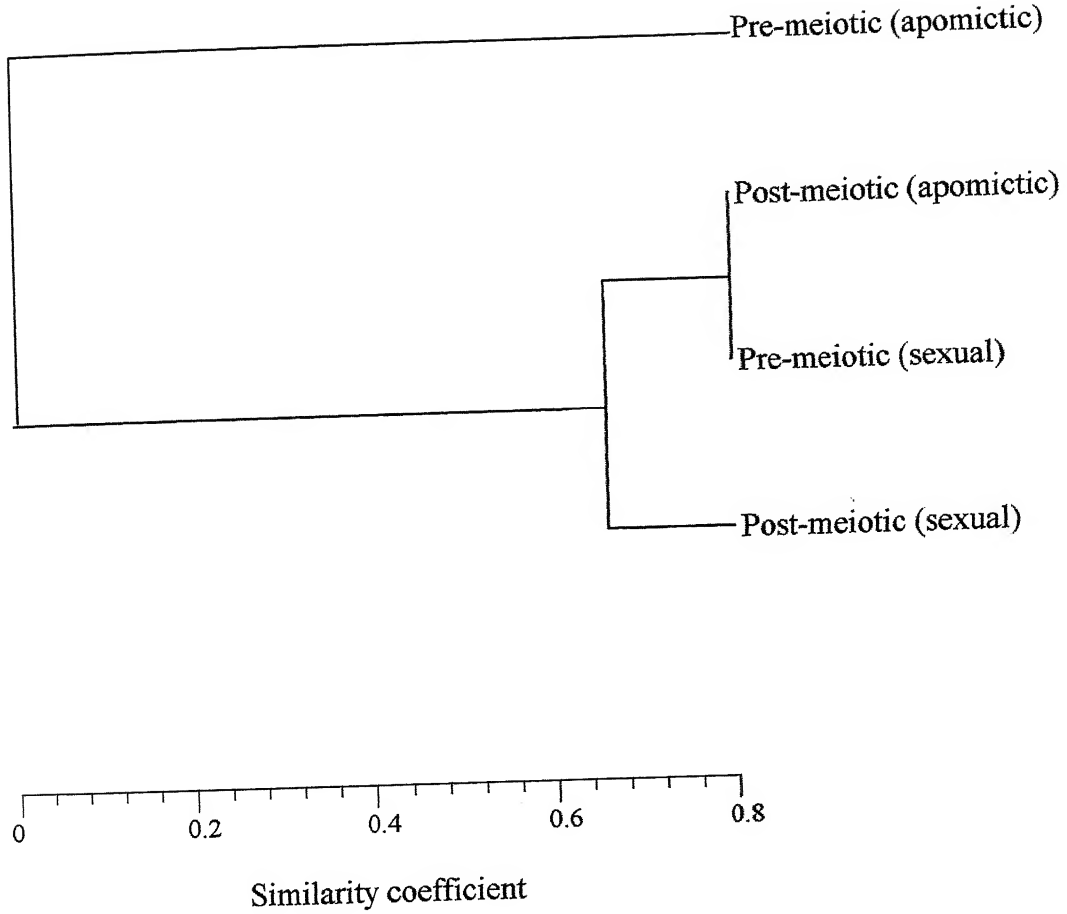


Fig. 7: UPGMA dendrogram depicting relationship among pre and post meiotic ovaries of apomictic and sexual plants based on isozyme analysis

recorded the least (24), (Table 5). Maximum polymorphism was observed for esterase enzyme followed by phosphoglucomutase. All the 6 alleles of Est-1 were associated with pre meiotic ovaries but were absent during pre meiotic sexual megasporogenesis. Three alleles were conspicuous as they were absent in ovaries of sexual plants altogether. Expression of isozymes can differentiate pre meiotic sexual from apomictic types as of the 14 loci, three loci viz. ACP-1, ACP-2 and AAT-2 did not reveal polymorphism. The Isozyme polymorphism and zymogram pattern are shown in Fig. 5 and Fig. 6.

4.4.2: Dendrogram analysis

Dendrogram analysis based on similarity of isozymes indicated grouping of pre and post meiotic sexual types and post meiotic apomictic types was distinct (Fig. 7). This clearly implied the stage dependent expression of isozymes and the possibility of specific isozymes associated with pre meiotic apomictic ovules. Isozymes seemed to play a role during early megasporogenesis than of later stages of embryo sac development.

4.5: DNA polymorphism using RAPD analysis

4.5.1: DNA polymorphism

DNA polymorphism was studied among sexual and apomictic F2 segregants using RAPD analysis. Bulked segregant analysis was carried out, using two bulks, apomict (five individuals) and sexual (eight individuals) F2 segregants, these DNA bulks were screened using 208 random decamer primers for identifying RAPD polymorphism. Forty-seven (22.5%) primers did not produce any amplified products. Twenty-five (12%) primers produced the amplified DNA fragment but the fragments were faint. One hundred and sixteen (55.7%) primers produced fragments that were monomorphic across two bulks. Twenty (9.6%) primers produced polymorphism (Table 6). The amplification pattern of these 19 primers (OPA-17, OPC-7, OPC-19, OPD-12, OPF-13, OPG-19, OPH-1, OPH-5, OPH-6, OPH-9, OPH-12, OPH-18, OPI-16, OPJ-15, OPK-2, OPK-8, OPK-18, OPK-19 and OPK-20) is shown in Fig. 8. When these 20 polymorphic primers were used in selected F2 segregants, fourteen (A17, C19, D12, F13, G19, H1, H5, H6, H12, H18, J15, K2, K18, and K19) did not show any clear cut differentiation pattern between apomictic and sexual F2 segregants, the amplification pattern from these primers in

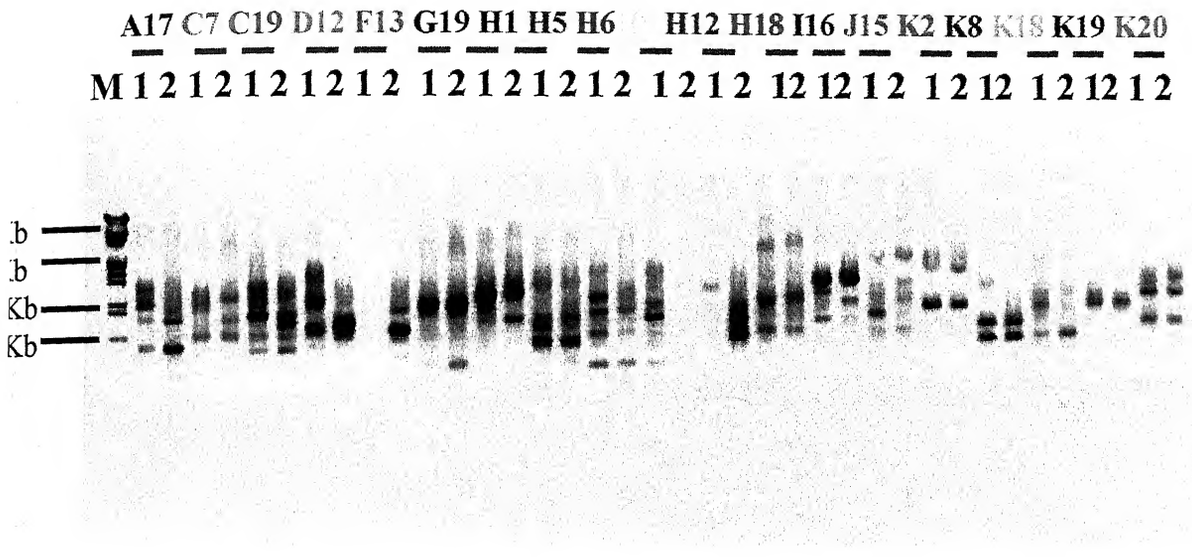


Fig. 8: Amplification profiles of apomictic and sexual bulks of F2 segregants of *Cenchrus ciliaris* with 19 selected reproducible polymorphic operon primers.

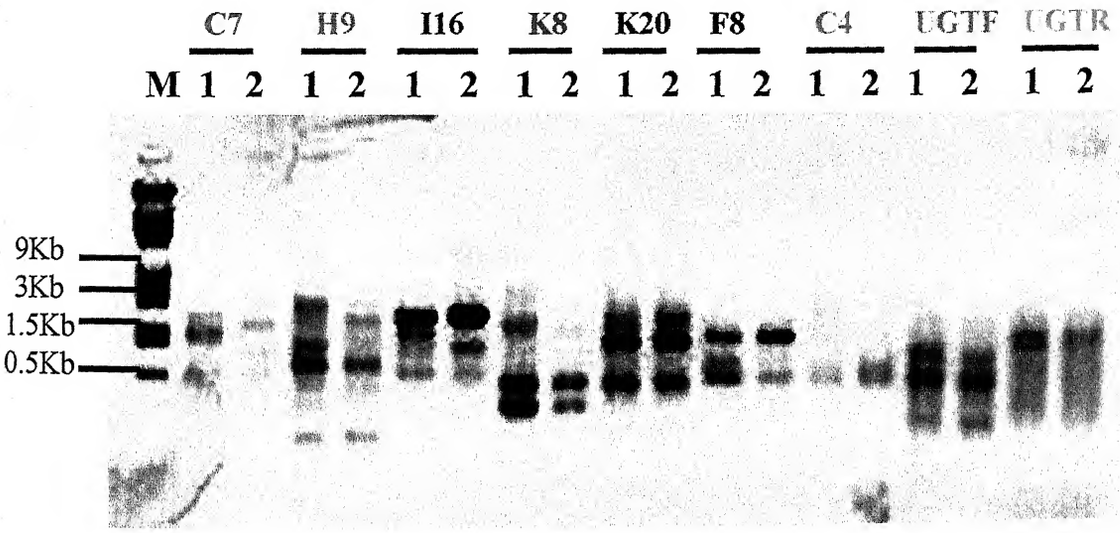


Fig. 9: Amplification profiles of apomictic and sexual bulk of F2 segregants of *Cenchrus ciliaris* with newly and previously reported polymorphic primers.

1: apomictic bulk

2: sexual bulk.

M: Lambda DNA/EcoRI+HindIII marker

Table 6. Screening of sexual and apomictic bulks of F2 segregants for selecting polymorphic primers

Total number of primers screened	208
Primers which did not amplified	47 (22.5%)
Primers which amplified faintly	25 (12%)
Primers which amplified monomorphic bands	116 (55.7%)
Primers which amplified polymorphic bands	20 (9.6%)
Primers finally selected for fingerprinting	6 (30%)

Table 7. Comparison of molecular markers in bulked apomictic and sexual F2 progeny

DNA	Marker (Primer bp)							
	UGT197 ~144bp	OPC4 ~600bp	OPF8 ~600bp	OPC7 ~1.5Kb	OPH9 ~800bp	OPI16 ~1Kb	OPK8 ~1Kb	OPK20 ~1Kb
Aposporous bulk	+	+	+	+	+	+	+	+
Sexual bulk	+	+	-	-	-	-	-	-

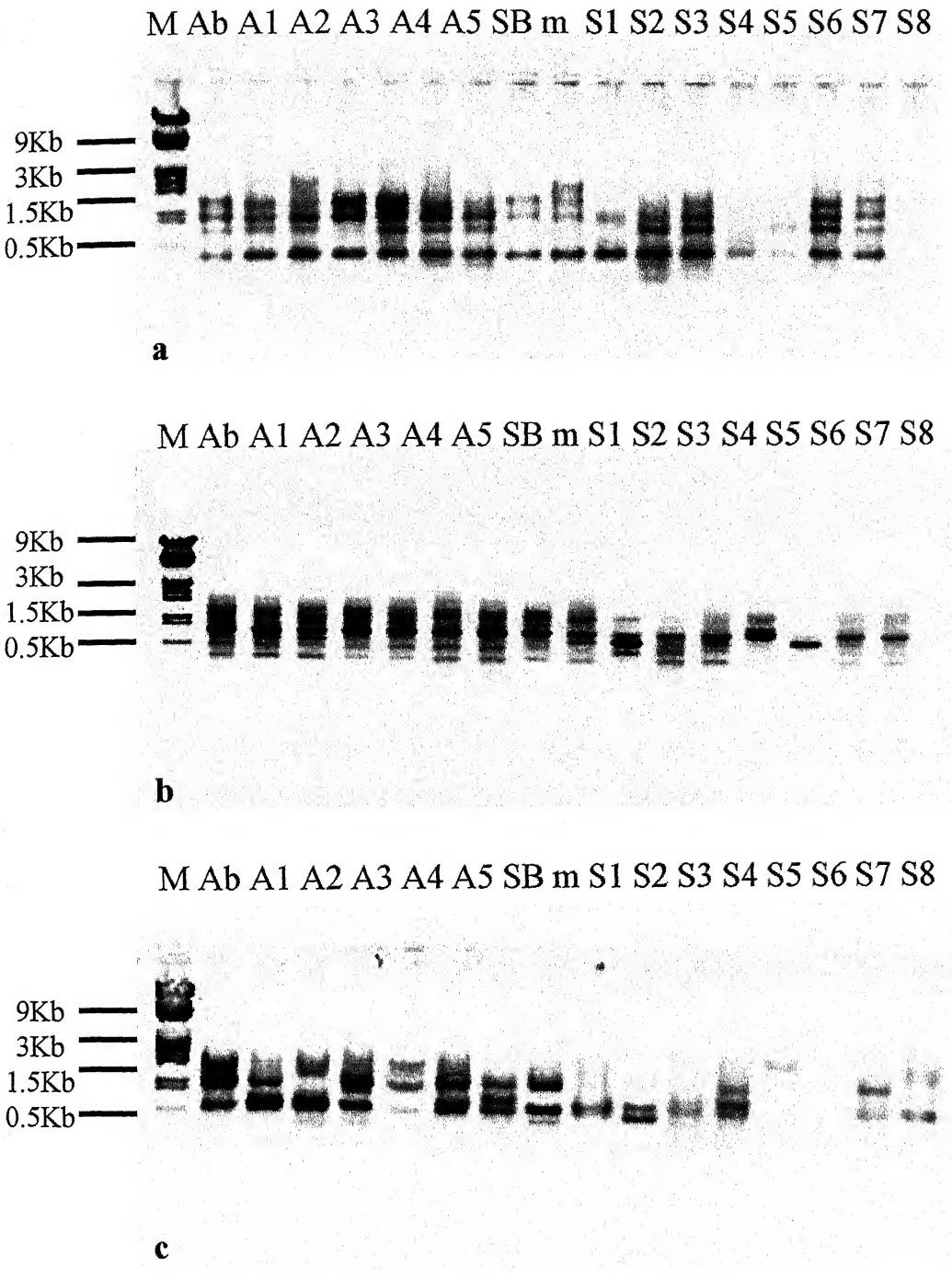


Fig.10:RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.10a by A17, Fig.10b by C19 and Fig.10c by D12 primers.

Lane A1 to A5: apomictic individuals
Lane S1 to S8: sexual individuals
m:sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M: Lambda DNA/EcoRI+HindIII marker

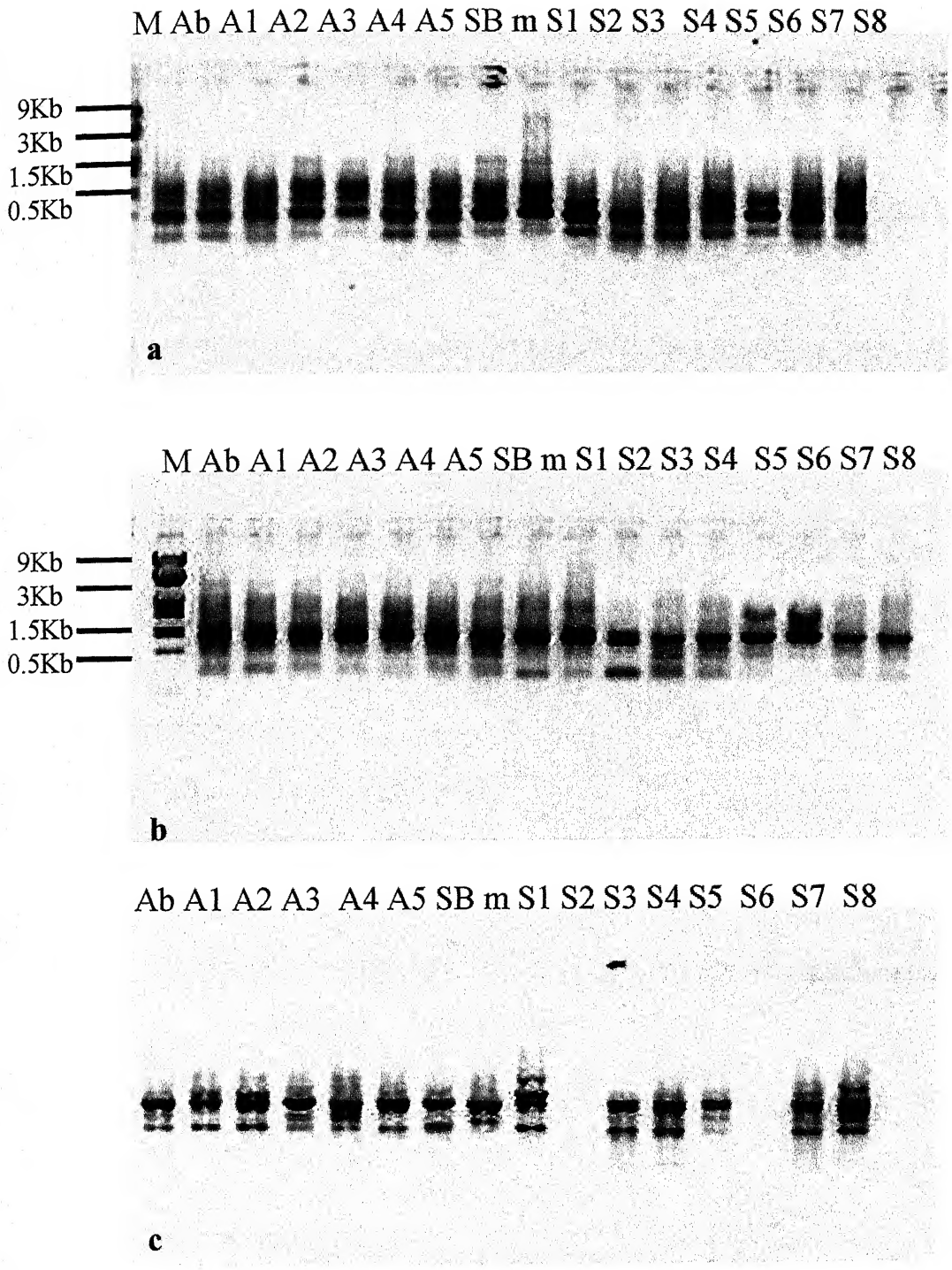


Fig.11: RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.11a by F13, Fig.11b by G19 and Fig.11c by H1 primers

Lane: A1 to A5 apomictic individuals
Lane: S1 to S8 sexual individuals
m: sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M: Lambda DNA/EcoRI+HindIII marker

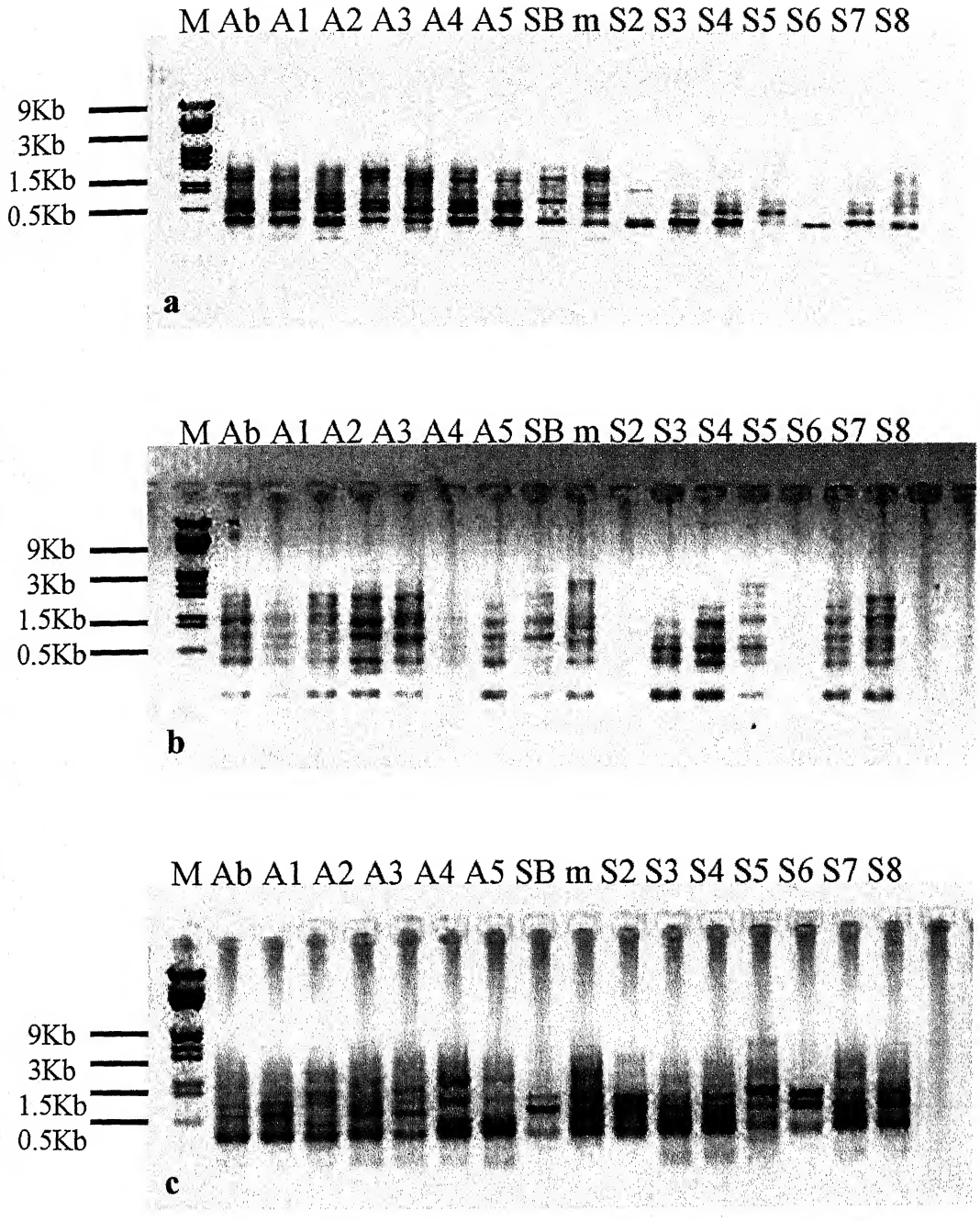


Fig.12: RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.12a by H5, Fig.12b by H6 and Fig.12c by H12 primers.

Lane A1 to A5: apomictic individuals
LaneS1 to S8: sexual individuals
m: sexual mother plant

Ab: apomictic bulk
Sb: sexual bulk
M: Lambda DNA/EcoRI+HindIII marker

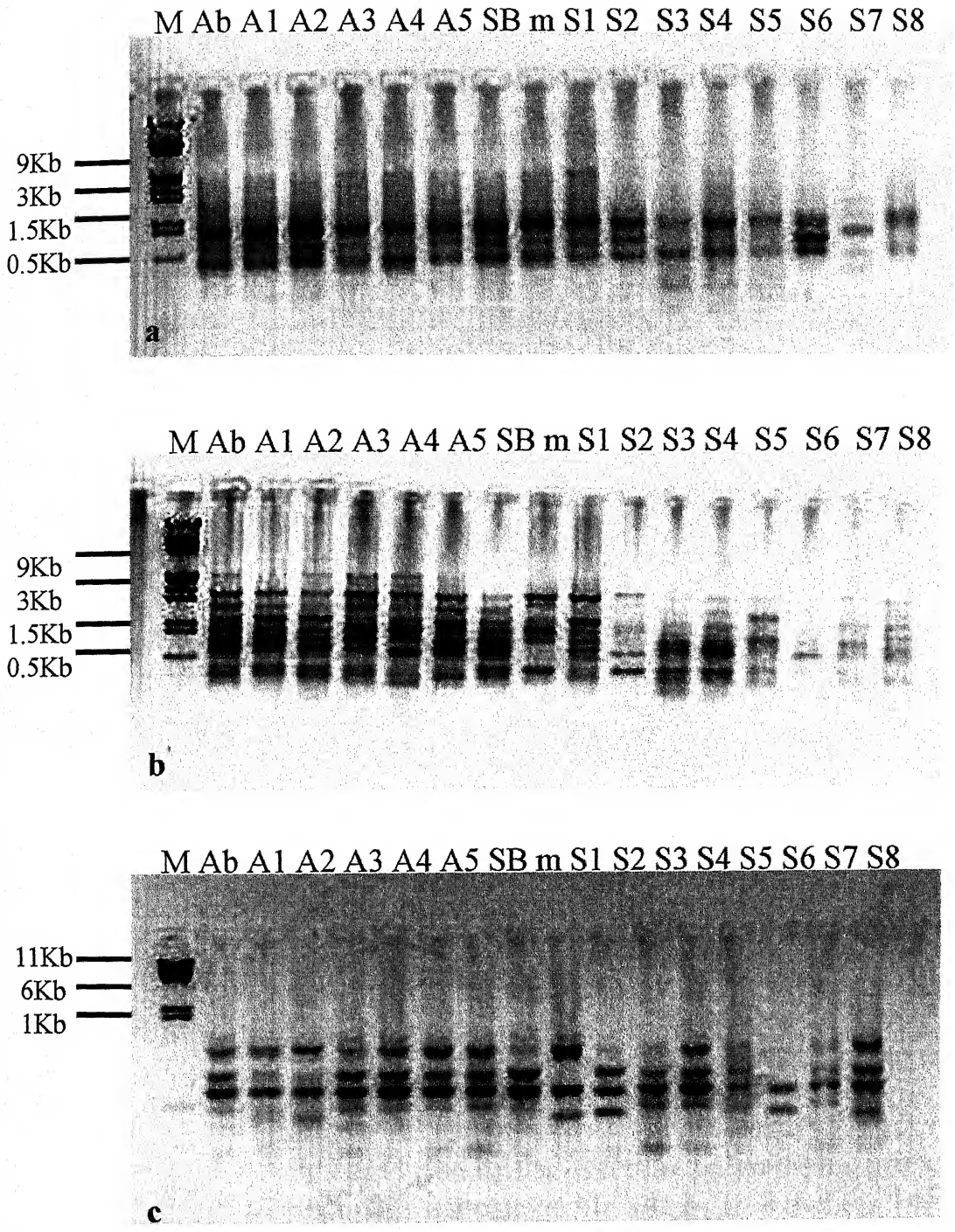


Fig.13: RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.13a by H18, Fig.13b by J15 and Fig.13c by K2 primers.

Lane A1 to A5: apomictic individuals
LaneS1 to S8: sexual individuals
m: sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M: Lambda DNA/EcoRI+HindIII marker

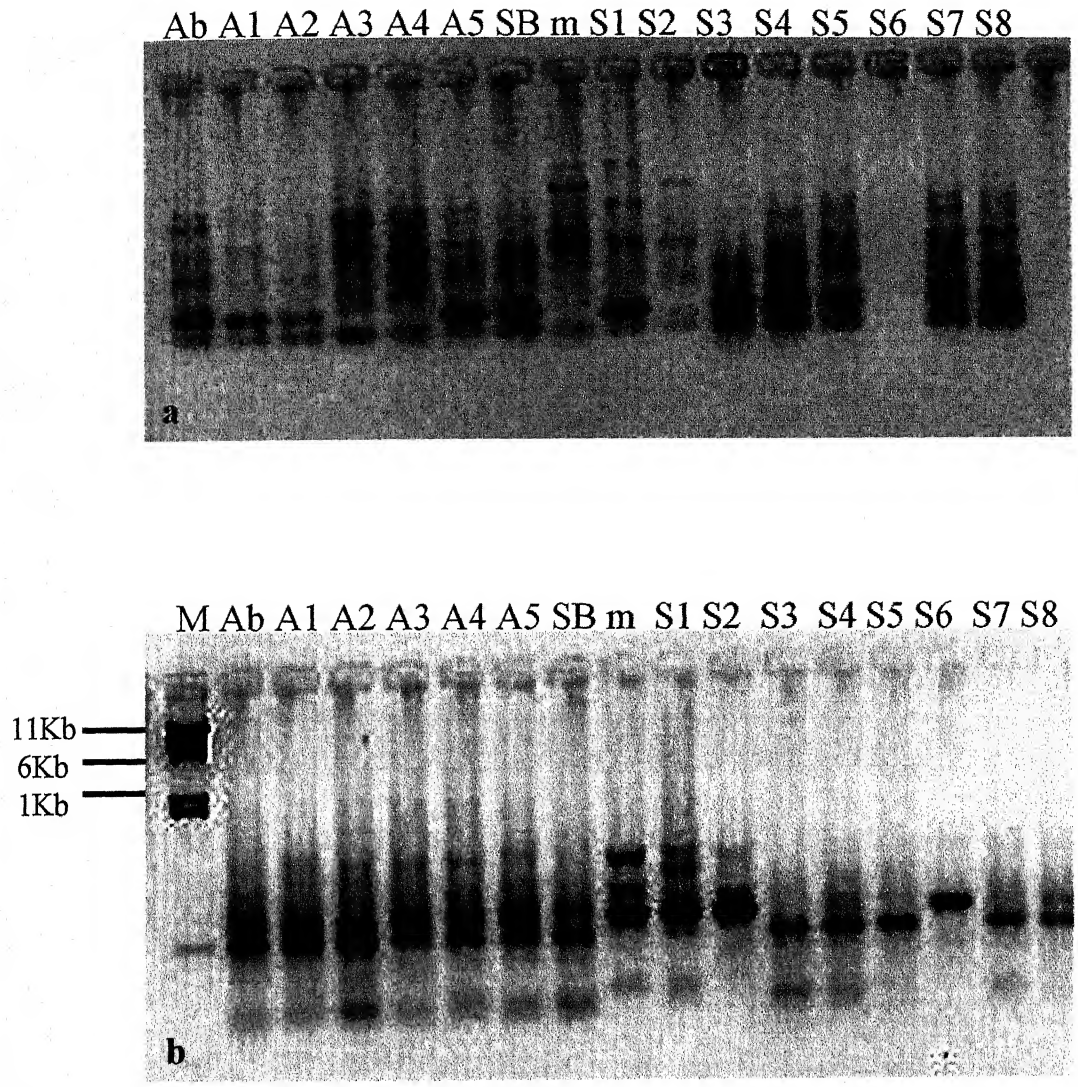


Fig.14: RAPD fingerprints produced by two different polymorphic primers in apomictic and sexual F2 segregants. Fig.14a by K18 and Fig.14b by K19 primers.

Lane A1 to A5: apomictic individuals
Lane S1 to S8: sexual individuals
m: sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M:Lambda DNA/HindIII marker.

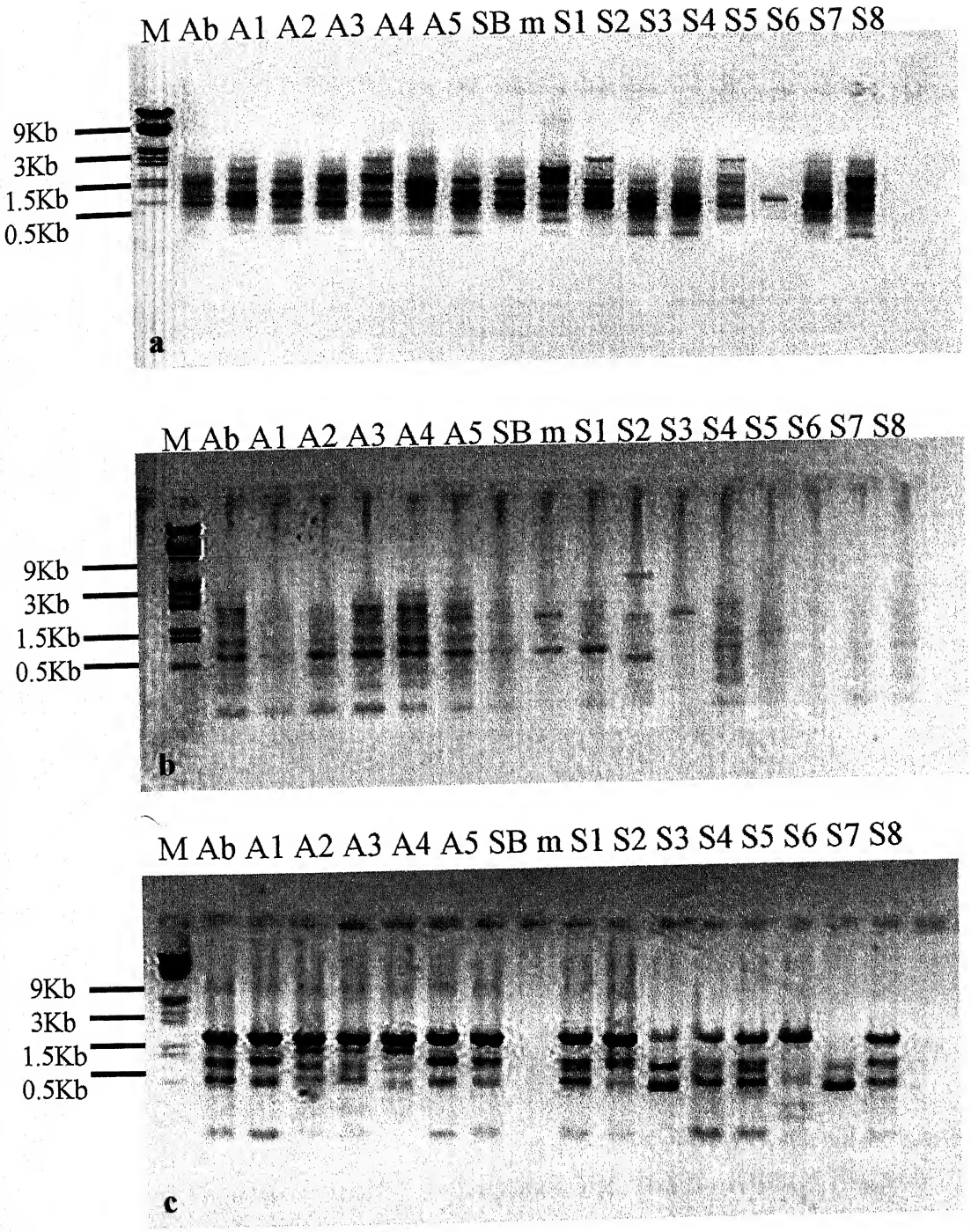


Fig.15: RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.15a by C7, Fig.15b by H9 and Fig.15c by I16 primers.

Lane A1 to A5 apomictic individuals
Lane S1 to S8 sexual individuals
m: sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M: Lambda DNA/EcoRI+HindIII marker

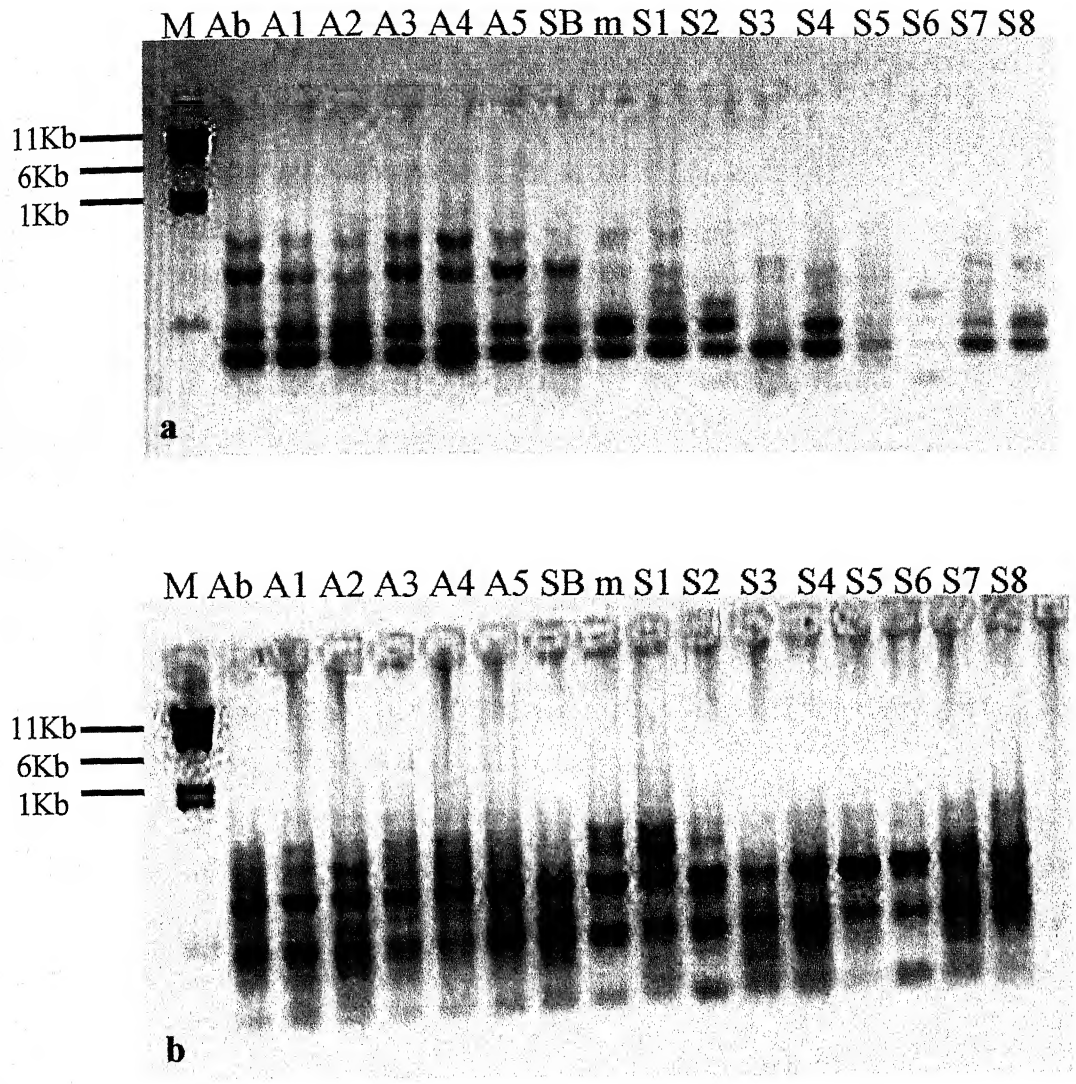


Fig.16: RAPD fingerprints produced by three different polymorphic primers in apomictic and sexual F2 segregants. Fig.16a by K8 and Fig.16b by K20 primers.

Lane A1 to A5 apomictic individuals
Lane S1 to S8 sexual individuals
m: sexual mother plant
Ab: apomictic bulk
Sb: sexual bulk
M:Lambda DNA/HindIII marker

mapping population are shown in Fig. 10a, b, c, Fig. 11a, b, c, Fig. 12a, b, c, Fig. 13a, b, c and in Fig. 14a, b, c. Only six primers could clearly exhibit polymorphism between sexual and apomictic segregants (Fig. 15a, b, c and Fig. 16a, b).

Previously reported markers linked to apomixis such as UGT-197 and OPC-04 were used for screening. There was no polymorphism observed among sexual and apomictic bulk, whereas the selected six polymorpic markers OPF-08 (600bp), OPC-07 (1.5Kb), OPH-09 (800bp), OPI-16 (1Kb), OPK-08 (1Kb), and OPK-20 (1Kb) gave poymorphism in apomictic and sexual bulk (Fig. 9). The size and polymorphic behavior of the previously reported and selected six-polymorpic marker are shown in Table 7.

Number of electrophoretic phenotypes (EPs) were analyzed among apomictic and sexual F2 segregants. All the polymorphic primers have exhibited larger number of EPs in sexual plants compared to the apomictic, which indicates higher amount of variation in sexual segregants. Primers OPC-07, OPH-05, OPH-9, OPJ-15, OPK-02, OPK-18 and OPK-20 have shown maximum number of EPs. A maximum of 5 EPs were observed in apomictic compared to 8 EPs in sexual types (Table 8).

Polymorphism information content (PIC) scores represent gene diversity for a specific locus. The higher the PIC scores for primer, the higher the probability for obtaining polymorphism using that primer. In our study, PIC scores for the RAPD primer pairs ranged from 0 to 1 (OPF-08) in apomicts, whereas in the sexual population, it ranged from 0 to 0.99 (Table 9). This indicated that the primer OPF-08 could be used for obtaining high level of polymorphism.

4.5.2: Population Genetic parameters

Various Population genetic parameters such as allele frequency, allelic diversity and richness, gene diversity, percent polymorphism and Shannon's information index were estimated. Gene frequencies were estimated for 36 loci (Table 10), generated from 6 polymorphic primers. A total of 72 alleles were identified among 13 individuals of the mapping population. While the mean number of alleles per locus varied from 1.33 (apomictic) to 1.69 (sexual) indicating the allelic richness, the effective number of alleles per locus, which is an indication of allelic evenness varied from 1.21 (apomictic) to 1.38 (sexual). Thus sexual population showed higher allelic diversity. Similarly gene diversity which is a measure of expected heterozygosity was higher in sexual population (0.22) compared to apomictic population (0.12). Shannon's genetic diversity index was also

Table 8. Electrophoretic phenotypes among apomictic and sexual F2 segregants exhibited by 17 polymorphic primers

Primer	Apomictic banding pattern	Sexual banding pattern	Total banding pattern
OPA17	2	5	5
OPC07	3	8	9
OPC19	2	6	8
OPF13	1	5	4
OPG19	3	6	6
OPH01	3	5	6
OPH05	3	6	9
OPH09	4	6	9
OPH18	1	4	4
OPI16	3	4	6
OPJ15	5	5	9
OPK02	4	7	9
OPK08	1	5	6
OPK18	4	7	9
OPK19	1	6	7
OPK20	3	6	9
OPF08	2	4	5

Table 9. Polymorphism information content calculated for apomictic (5 individuals) and sexual (8 individuals) F2 segregants for 36 loci

S.N.	Locus	PIC	
		Apomictic	Sexual
1	OPC07-1a	-	0.5
2	OPC07-1b	0	0.9
3	OPC07-2a	0.2	0.37
4	OPC07-2b	0.99	0.957
5	OPC07-3a	-	0.75
6	OPC07- 3b	0	0.75
7	OPC07-4a	-	-
8	OPC07-4b	0	0
9	OPC07-5a	-	0.875
10	OPC07-5b	0	0.583
11	OPC07-6a	0.6	0.63
12	OPC07-6b	0.86	0.85
13	OPH09-1a	0	0.13
14	OPH09-1b	-	0.99
15	OPH09-2a	0.6	0
16	OPH09-2b	0.89	-
17	OPH09-3a	0.80	0.25
18	OPH09-3b	0.7	0.98
19	OPH09-4a	0.80	0.13
20	OPH09-4b	0.7	0.99
21	OPH09-5a	-	0.13
22	OPH09-5b	0	0.99
23	OPH09-6a	0.6	0.13
24	OPH09-6b	0.89	0.99
25	OPH09-7a	-	0.37
26	OPH09-7b	0	0.957
27	OPI16-1a	0.2	0
28	OPI16-1b	0.99	-
29	OPI16-2a	-	0.75
30	OPI16-2b	0	0.75
31	OPI16-3a	0	0
32	OPI16-3b	0.95	-
33	OPI16-4a	-	-
34	OPI16-4b	0	0
35	OPI16-5a	-	0.75
36	OPI16-5b	0	0.75
37	OPI16-6a	0	0.13

38	OPI16-6b	-	0.99
39	OPK08-1a	-	0
40	OPK08-1b	0	-
41	OPK08-2a	-	0
42	OPK08-2b	0	-
43	OPK08-3a	0	0.37
44	OPK08-3b	-	0.957
45	OPK08-4a	-	0.63
46	OPK08-4b	0	0.85
47	OPK08-5a	-	0.75
48	OPK08-5b	0	0.75
49	OPK20-1a	0	0.5
50	OPK20-1b	-	0.9
51	OPK20-2a	0.80	0.25
52	OPK20-2b	0.70	0.98
53	OPK20-3a	-	-
54	OPK20-3b	0	0
55	OPK20-4a	0.2	0.13
56	OPK20-4b	0.99	0.99
57	OPK20-5a	-	-
58	OPK20-5b	0	0
59	OPK20-6a	0.2	0.63
60	OPK20-6b	0.99	0.85
61	OPK20-7a	-	-
62	OPK20-7b	0	0
63	OPF08-1a	-	0.875
64	OPF08-1b	0	0.583
65	OPF08-2a	-	0.13
66	OPF08-2b	0	0.99
67	OPF08-3a	-	0.875
68	OPF08-3b	0	0.583
69	OPF08-4a	0.6	0
70	OPF08-4b	0.86	-
71	OPF08-5a	0	0.13
72	OPF08-5b	1	0.99

a: denotes first allele

b: denotes second allele

Table10. Binary data scored as 0 (absence) and 1 (presence) generated from 6 primers in apomictic and sexual F2 segregants including the sexual mother parent

	Primer C7	Primer H9	Primer I16	Primer K8	Primer K20	Primer F8
Ab	101110	0111111	011110	11011	0110101	11100
A1	111110	0000101	010110	11011	0110101	11110
A2	101111	0011111	010110	11011	0111101	11100
A3	101111	0111101	011110	11011	0110101	11100
A4	101111	0111111	011110	11011	0110101	11110
A5	101110	0111111	110110	11011	0010111	11110
Sb	001111	0000100	010110	01011	0011111	10101
M	001110	0010100	010010	00011	1110101	00000
S1	011111	0000101	010110	00111	1110111	10100
S2	101110	1010000	000100	00111	1110101	10100
S3	001111	0010000	010110	00001	0010111	10100
S4	000111	0001011	010110	00011	0010111	10100
S5	111110	0000000	010101	00000	0010101	10100
S6	000110	0000000	000110	00100	0010101	10101
S7	101101	0000001	010110	00011	1010111	11100
S8	111111	0000000	010110	00011	1011111	00000

Table 11. Gene frequency across 72 alleles of 36 loci for apomictic and sexual F2 segregants

S.N.	Locus	Gene Frequency	
		Apomictic	Sexual
1	OPC07-1a	-	0.71
2	OPC07-1b	1.00	0.29
3	OPC07-2a	0.89	0.79
4	OPC07-2b	0.11	0.21
5	OPC07-3a	-	0.5
6	OPC07- 3b	1.00	0.5
7	OPC07-4a	-	-
8	OPC07-4b	1.00	1.00
9	OPC07-5a	-	0.35
10	OPC07-5b	1.00	0.64
11	OPC07-6a	0.63	0.61
12	OPC07-6b	0.36	0.38
13	OPH09-1a	1.00	0.93
14	OPH09-1b	-	0.064
15	OPH09-2a	0.63	1.00
16	OPH09-2b	0.34	-
17	OPH09-3a	0.44	0.86
18	OPH09-3b	0.55	0.134
19	OPH09-4a	0.44	0.93
20	OPH09-4b	0.55	0.064
21	OPH09-5a	-	0.935
22	OPH09-5b	1.00	0.064
23	OPH09-6a	0.63	0.935
24	OPH09-6b	0.36	0.064
25	OPH09-7a	-	0.7906
26	OPH09-7b	1	0.2094
27	OPI16-1a	0.89	1.00
28	OPI16-1b	0.10	-
29	OPI16-2a	-	0.50
30	OPI16-2b	1.00	0.50
31	OPI16-3a	0.77	1.00
32	OPI16-3b	0.22	-
33	OPI16-4a	-	-
34	OPI16-4b	1.00	1.00
35	OPI16-5a	-	0.50
36	OPI16-5b	1.00	0.50
37	OPI16-6a	1.00	0.935

38	OPI16-6b	-	0.064
39	OPK08-1a	-	1.00
40	OPK08-1b	1.00	-
41	OPK08-2a	-	1.00
42	OPK08-2b	1.00	-
43	OPK08-3a	1.00	0.7906
44	OPK08-3b	-	0.2094
45	OPK08-4a	-	0.612
46	OPK08-4b	1.00	0.387
47	OPK08-5a	-	0.500
48	OPK08-5b	1.00	0.500
49	OPK20-1a	1.00	0.7071
50	OPK20-1b	-	0.2929
51	OPK20-2a	0.44	0.8660
52	OPK20-2b	0.55	0.1340
53	OPK20-3a	-	-
54	OPK20-3b	1.00	1.00
55	OPK20-4a	0.89	0.9354
56	OPK20-4b	0.10	0.0646
57	OPK20-5a	-	-
58	OPK20-5b	1.00	1.00
59	OPK20-6a	0.89	0.6124
60	OPK20-6b	0.10	0.3876
61	OPK20-7a	-	-
62	OPK20-7b	1.00	1.00
63	OPF08-1a	-	0.3536
64	OPF08-1b	1.00	0.6464
65	OPF08-2a	-	0.9354
66	OPF08-2b	1.00	.06446
67	OPF08-3a	-	0.3536
68	OPF08-3b	1.00	0.6464
69	OPF08-4a	0.6325	1.00
70	OPF08-4b	0.3675	-
71	OPF08-5a	1.00	09354
72	OPF08-5b	-	0.646

a: denotes first allele

b: denotes second allele

higher in sexual segregants (0.33) compared to apomictic segregants (0.18), (Table 11 and Table 12). Overall, there were more number of polymorphic loci (25) accounting for 69.44 percent polymorphism in the sexual population compared to apomictic population (12 loci accounting for 33.3 percent polymorphism).

4.5.3: Dendrogram analysis

The dendrogram analysis carried out using binary data of the polymorphic bands between five apomicts, eight sexual individual of the mapping population and sexual mother plant indicated two clearly distinguishing clusters, one comprising of apomictic individuals and another with sexual individuals. These results were obtained using similarity indices, which implied that the each cluster consisted of individuals, which shared similar bands. (Fig. 17). The similarity indices varied from 0.44 (between A4 and S6) to 0.944 (between A4 and A3). The dendrogram indicated apomictic and sexual bulks in the respective groups. Maximum similarity was observed among apomictic individuals than among sexual individuals. The F2 segregants from the same half-sibs have shared maximum similarity both among sexual and apomictic progenies. For example while A3 and A4 showed similarity indices of 0.944, the sexual segregants such as S3 and S4 were most similar (0.833). (Table 13)

4.6: Development of SCAR marker

4.6.1: Identification of polymorphic RAPD fragment

From the previous RAPD data it is clear that the primer OPF-08 exhibited clear cut polymorphism between apomictic and sexual bulk and one bright and reproducible band approximately 600bp in size was amplified only with apomictic bulk. RAPD PCR amplification with eight sexual and five apomictic F2 segregants as well as their bulks along with their sexual mother plant was performed with the polymorphic primer OPF-08 (Fig. 18). All the apomicts F2 segregants produced 3 to 4 bands including 600bp polymorphic fragment, whereas in the sexual individuals and in the mother plant this polymorphic band was absent, except in one sexual plant.

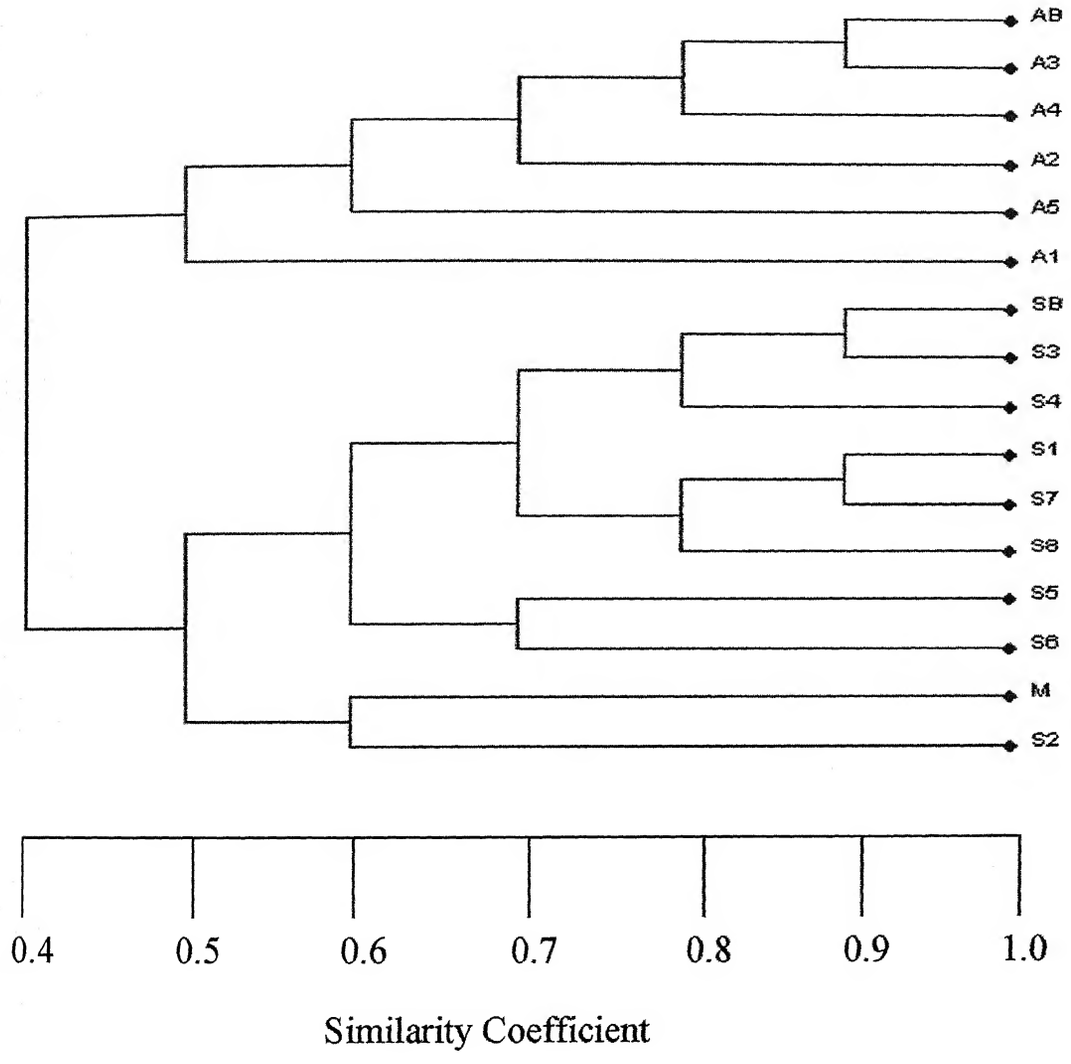


Fig.17: UPGMA Dendrogram of apomictic and sexual F2 segregants including their bulks, and sexual mother plant. The scale is based on coefficient of similarity of RAPD bands.

Table12. Population genetic analysis of apomictic and sexual F2 segregants using POPGENE version 1.31

S.N.	Observations	Apomictic (mean)	Sexual (mean)
1.	Observed number of alleles per locus	1.333 (+ - 0.4781)	1.6944 (+ - 0.4672)
2.	Effective number of alleles	1.2188 (+ - 0.3653)	1.3845 (+ - 0.3901)
3.	Gene diversity	0.1235 (+ - 0.1937)	0.2236 (+ - 0.2000)
4.	Information index	0.1827 (+ - 0.2771)	0.3382 (+ - 0.2776)
5.	Number of polymorphic loci	12	25
6.	Percentage polymorphism	33.33%	69.44%

Table 13. Similarity indices among 5 apomicts, 8 sexual F2 segregants, their bulks including sexual mother plant estimated from amplified fragment data

	AB	A1	A2	A3	A4	A5	SB	M	S1	S2	S3	S4	S5	S6	S7	S8
AB	1.000															
A1	0.806	1.000														
A2	0.889	0.806	1.000													
A3	0.944	0.806	0.889	1.000												
A4	0.944	0.806	0.889	0.944	1.000											
A5	0.861	0.778	0.806	0.806	0.861	1.000										
SB	0.611	0.694	0.722	0.667	0.611	0.639	1.000									
M	0.639	0.667	0.639	0.639	0.583	0.556	0.694	1.000								
S1	0.611	0.750	0.667	0.667	0.611	0.583	0.778	0.750	1.000							
S2	0.611	0.639	0.611	0.611	0.556	0.528	0.611	0.750	0.722	1.000						
S3	0.611	0.639	0.667	0.667	0.611	0.639	0.833	0.750	0.778	0.722	1.000					
S4	0.667	0.639	0.722	0.667	0.667	0.694	0.778	0.639	0.778	0.611	0.833	1.000				
S5	0.556	0.694	0.556	0.556	0.500	0.528	0.667	0.639	0.667	0.722	0.778	0.667	1.000			
S6	0.500	0.583	0.500	0.500	0.444	0.472	0.722	0.639	0.667	0.722	0.778	0.722	0.778	1.000		
S7	0.639	0.722	0.694	0.694	0.639	0.667	0.750	0.667	0.806	0.694	0.806	0.806	0.694	0.639	1.000	
S8	0.500	0.639	0.611	0.556	0.500	0.528	0.778	0.750	0.778	0.667	0.778	0.722	0.722	0.611	0.806	1.000

AB: Apomictic bulk
SB: Sexual bulk
A1 to A5: Apomictic individuals
S1 to S8: Sexual individuals
M: Sexual mother plant

4.6.2: Cloning and Sequencing of polymorphic RAPD band

The amplified product (600bp) of OPF-08 primer was cloned in pGEMT-Easy cloning vector and the cloned insert was checked by EcoRI digestion (Fig. 22a, b). The identity of the cloned product was verified by hybridization of the fragment to Southern blot of amplified product (RAPD product amplified from OPF-08 primer) of randomly chosen F2 (3 apomicts and 5 sexual) segregants. The hybridization pattern of 3 apomicts (A1, A2 and A3) and 5 sexual (S1, S2, S3, S4 and S5) is shown in Fig. 20. The cloned 600bp fragment which was restricted and used as probe showed the strong hybridization signal in apomictic individuals, whereas no hybridization signal were observed in sexual individuals, verified that the PCR product wase homologous to the DNA clone.

The cloned amplified product was sequenced from each end (T7 and SP6 promoter site). The sequence was subjected to Blast analysis for searching homology to known and unknown Gene/Protein in the Gene Bank. Maximum homology was found to be with the *Pennisetum glaucum* isolate RGPM and *Zea Mays* T cytoplasm male sterility.

4.6.3: Amplification of genomic DNA using SCAR primers

For cloned RAPD product, a forward 23-mers and reverse 24-mer SCAR primers were synthesized based on sequence data. PCR amplification was performed with these primers at an annealing temperature of 63°C, using genomic DNA as a template from the F2 segregating mapping population. An approximately 600bp amplicon was amplified from all the apomictic individuals and one of the sexual individuals (Fig. 19). SCAR description, i.e. sequence of the primers, size of the primer, Tm, GC content of the primers and amplified product is shown in the Table 14.

4.6.4: Southern analysis of SCAR marker

To characterize the SCAR marker, the Southern hybridization was carried out. Membrane was prepared with the total genomic DNA and was digested with DraI restriction enzyme from genotype combinations such as A1S1, A2S2, A3S3 and A4S4 pair wise and probed with 600 bp eluted fragment. There was no hybridization signal in all the types of sexual individuals, whereas two bands were observed in apomictic individuals. The two bands were of 1.2Kb and 1.8Kb size in apomictic individuals. (Fig.

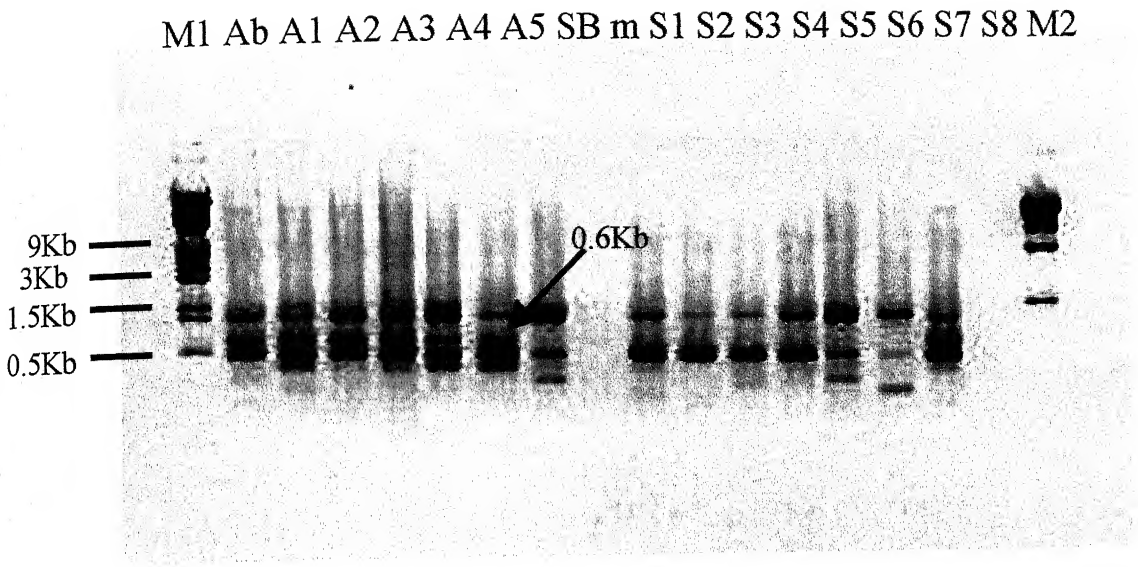


Fig.18: RAPD fingerprints produced by arbitrary primer OPF-08 in apomictic and sexual F2 segregants.

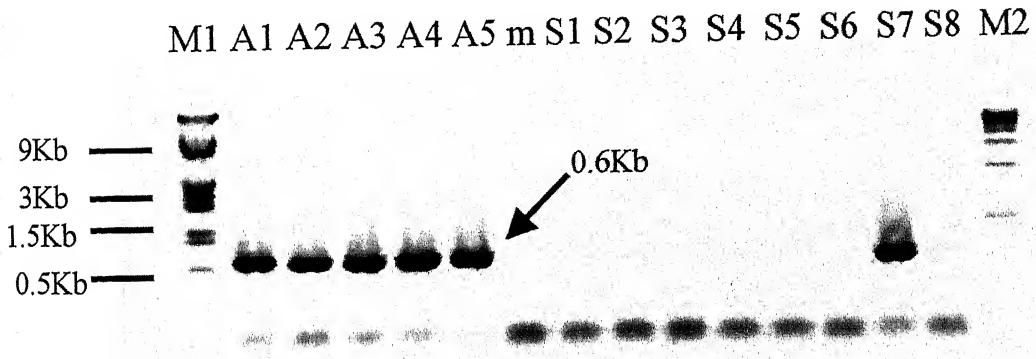


Fig.19: Amplification of genomic DNA of apomictic and sexual F2 segregants using SCAR primers.

Lane A1 to A5: apomictic individuals
Lane S1 to S8: sexual individuals
m: sexual mother plant.
M1: Lambda DNA/EcoRI+HindIII marker
M2: Lambda DNA/HindIII marker
Arrow indicate the 0.6Kb polymorphic amplified fragments

Table 14: Description of SCAR marker Apo-F8 closely associated with apomixis in *C. ciliaris*

SCAR marker name	Primer sequence forward and reverse (5-3)	Size bp of primers	Tm of the primers	Optimal annealing	C+G% of the primers	Amplified Product
ApoF8 SCAR	CAATGTCCA GGACTCCTT TTTGG	23bp	68°C	63°C	47.8%	~600bp
	AGGTGATAG GGGAATTGC TAAAGT	24bp	68°C		41.6%	

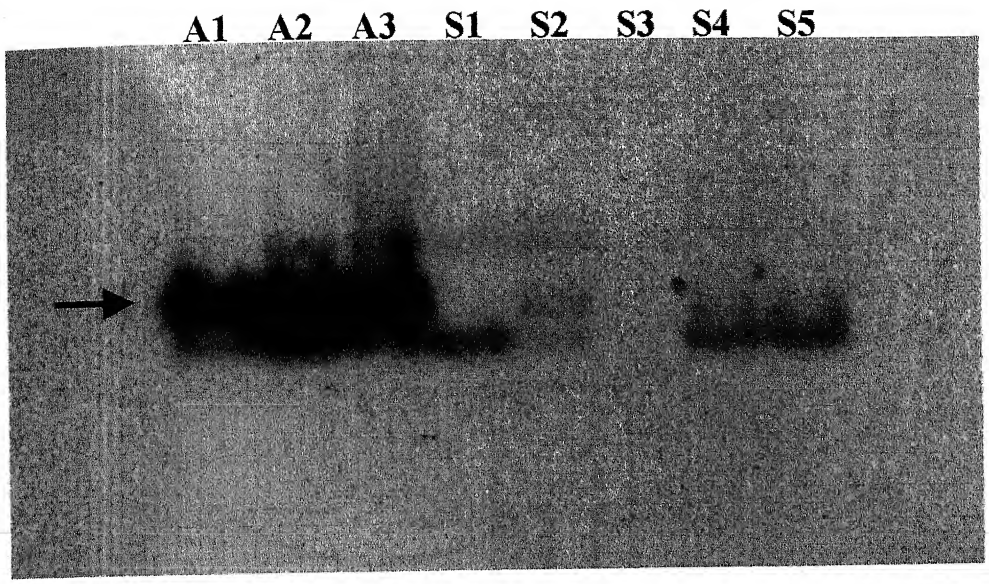


Fig.20: Southern hybridization of apomictic and sexual individuals.PCR product was transferred to the membrane and hybridized with 600bp eluted RAPD fragment.
LaneA1,A2,A3: apomictic individuals
LaneS1,S2,S3,S4,S5: sexual individuals.
Arrow indicate the hybridization signal

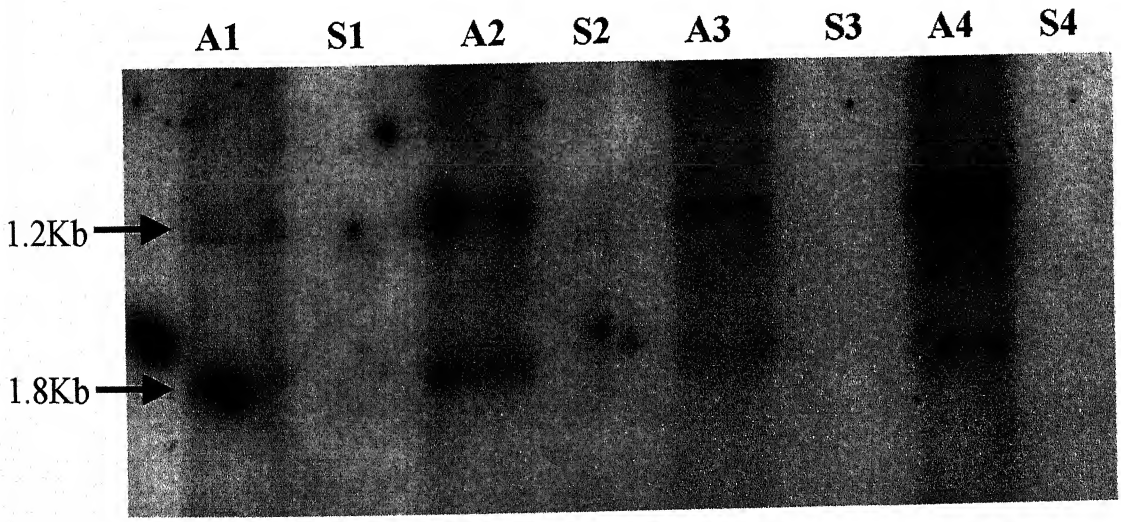


Fig.21: Restriction fragment length polymorphism (RFLP) of F2 population of apomictic and sexual segregants of *Cenchrus Ciliaris*, using ApoF8 as a probe on DraI digested DNA.
Lane A1,A2,A3,A4: apomictic individuals
Lane S1,S2,S3,S4: sexual individuals.
Arrow indicate the hybridization signal of 1.2Kb and 1.8Kb.

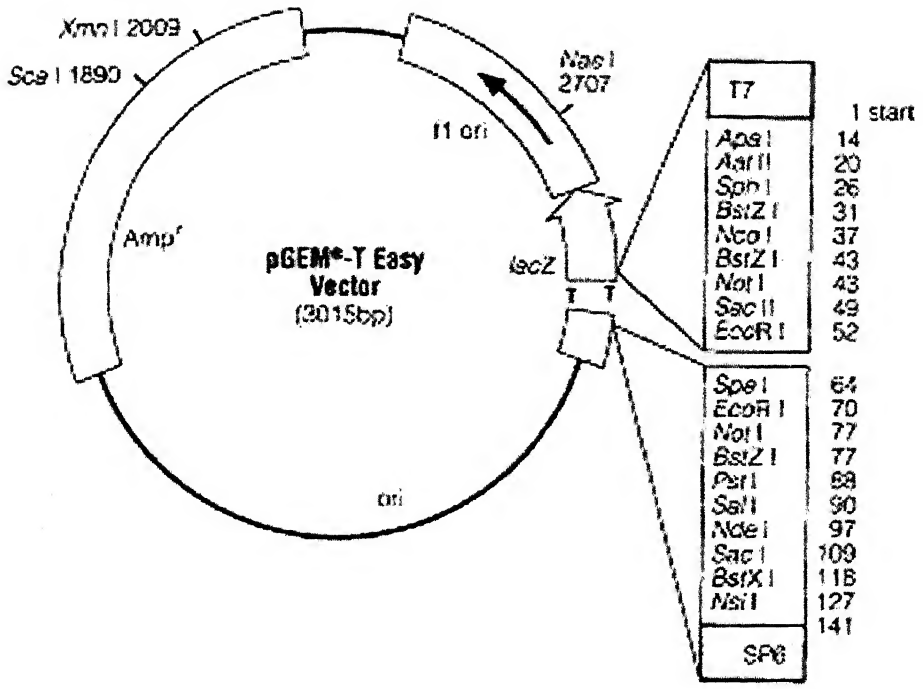


Fig. 22a: pGEM-T Easy plasmid vector showing multiple cloning sites

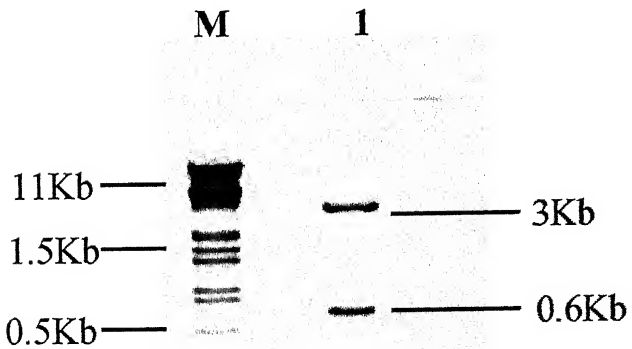


Fig. 22b: Restriction digestion of cloned ApoF08.
M: Lambda DNA/EcoRI+HindIII marker
1: EcoRI restricted plasmid DNA

21). These two fragments were eluted from gel on the basis of their size and PCR amplification was performed with SCAR specific primers a 600 bp amplicon was produced in both cases.

4.7: Isolation of cDNA fragments associated with apomixis by subtractive hybridization

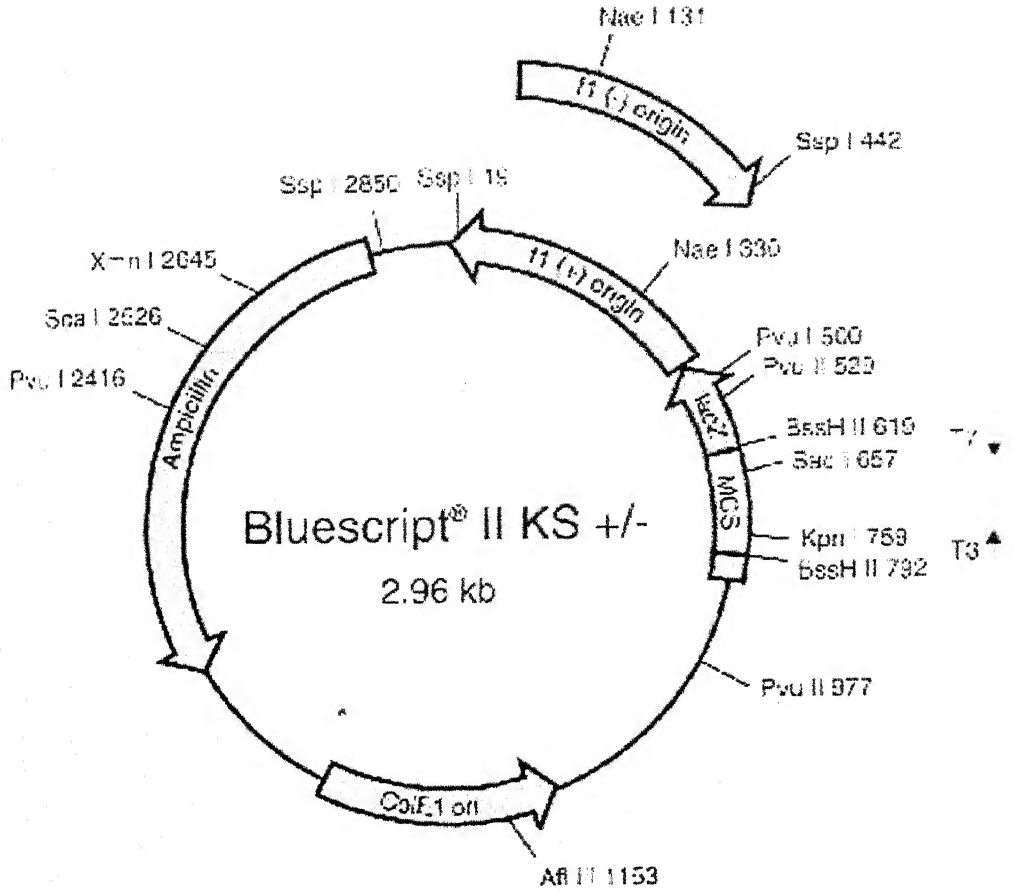
4.7.1: Subtractive hybridization

The floral buds were selected based on the extent of stigma exertion, which were indicative of stage of embryo sac development. The floral buds were pooled in such a way that different stages of embryo sac development was covered. Through subtractive hybridization technique a total of 141 cDNA fragments were cloned into pBluescript cloning vector (Fig. 23), which ranged in size from 100bp to 1Kb, cloned inserts were checked by colony PCR and were sequenced using shot gun sequencing approach.

4.7.2: Sequence analysis

141 cDNA fragments were classified into large and small sized fragments and only longer fragments were subjected to BLAST analysis for searching homology to known and unknown Gene/Protein in the GeneBank. BLAST results have indicated different families of proteins (Table 15 and in Table 16), which formed the basis of selecting appropriate clones for further bioinformatic analysis. Clones such as 2H2 and 1B8 showed homology to cdc-2 like protein kinase and bZIP family transcription factors respectively while clones 2A6 and 1A4 indicated homology to receptor like protein kinases. While 1E12 clone showed homology to Histone H2A protein, 1H8 and 1E3 showed homology to unknown proteins. 1C10 did not show any significant hit.

To understand phylogenetic relationships among these selected clones with SCAR (Fig. 27), CLUSTAL W analysis was carried out with the help of DNASTAR 4.05. The results indicated that, all the four-protein kinases clustal together, while bZIP protein was found to close to pistil extensin protein and an unknown putative protein of rice. The cytoplasm male sterility factor as indicated by SCAR marker ApoF-08 was found to be close to unknown protein of rice and to putative histone H2A of rice. This



T3 Promoter	KpnI **ApaI** XhoI SalI HindIII PstI BamHI XbaI **NotI** SacII SacI	T7 Promoter

MCS(657bp-759bp)

Fig.23: pBluescript II KS +/- phagemid vector

Table15. Putative genes and proteins which showed homology to the cDNA sequences isolated from *C. ciliaris* obtained from BLASTX analysis

Clone	Direct protein by BLASTX				
	ID	Name	E-value	Length % id	Score
2F4	AAK61381	Pistil extensin like protein.	4e-13	64/185=34%	77
1E3	AAU92216	Unknown protein (hypothetical protein) *Oryza sativa.*	2e-68	124/141=87%	259
1E12	XP_469689	Putative histone H2A protein *Oryza sativa.*	2e-20	50/53=94%	99
1F1	NP_921528	Putative centromere protein *Oryza sativa.*	2e-77	147/189=77%	290
1F12	AAP44746	Putative extensin (hypothetical) protein.	0.39	27/64=42%	37
1B8	AAU94427	Putative bZIP family transcription factor *Arabidopsis thaliana.*	4e-32	73/124=58%	139
1H8	AAT93853	Unknown protein *Oryza sativa.*	2e-08	26/28=92%	60
1A4	BAB02861	Putative protein kinase (receptor-like protein kinase) *Oryza sativa*	2e-52	100/130=76%	207
2H2	AAO00925	Cdc2-like protein kinase *Arabidopsis thaliana.*	8.6	18/50=36%	33
2A6	CAD41324	Putative protein kinase (receptor-like protein kinase) *Oryza sativa.*	1e-50	97/130=74%	201
1C10		No significant hit			

Table16: Putative genes and proteins which showed homology to the cDNA sequences isolated from *C. ciliaris* obtained from BLASTN analysis

Clone	Est match by BLASTN				
	ID	Name	E-value	Length % id	Score
2F4	21741695	*Oryza sativa* genomic DNA chromosome 4 BAC clone	8e-52	233/273=84%	212
1E3	21210388	*Zea mays* PC0079728 mRNA sequence	e-132	358/397=90%	478
1E12	21207134	*Zea mays* PC0122239 mRNA sequence	1e-46	146/162=90%	194
1F1	21207212	*Zea mays* PC0142944 mRNA sequence	e-145	486/560=86%	523
1F12	21211013	*Zea mays* PC0066270 mRNA sequence	1e-26	129/146=88%	129
1B8	21211290	*Zea mays* PC0110549 mRNA sequence	2e-99	293/325=90%	371
1H8	21212674	*Zea mays* PC0142361 mRNA sequence	5e-06	66/78=84%	60
1A4	21741695	*Oryza sativa* genomic DNA BAC clone	8e-52	233/273=84%	212
2H2	1684627	Artificial sequence	3e-64	164/172=95%	254
2A6	21741695	*Oryza sativa* genomic DNA chromosome 4 BAC clone	2e-49	232/273=84%	212
1C10	19851516	*Sorghum bicolor* clone BAC 10J22	1e-50	177/200=88%	206

indicated the possible similar functions associated with flower development, since it was close to pistil extensin protein.

4.7.3: Northern analysis

Specificity of the 16 selected clones was checked by northern analysis (Table17). Total RNA from apomictic and sexual ovaries was blotted on membrane and probed with labeled cDNA fragments. The northern profiles were shown in Fig. 24a and b and in Fig. 25a and b. Four clones (1A4, 2A6, 1G1 and 2H2) hybridized with RNA from ovaries of both apomictic and sexual plant. Clone 1B8 showed a more intense hybridization with ovaries of only apomictic plant but not with sexual plant RNA. (Fig. 26a) Due to complete absence of signal in the sexual ovaries the blot was again stripped and reprobed with control 26srRNA to confirm the presence of RNA in sexual plant samples. The control gave hybridization signals in both the apomictic and sexual ovaries. (Fig. 26b)

4.7.4: Characterization of cDNA fragments

To characterize 11 clones, internal primers were designed for each clone using primer design programme of DNASTAR 4.05. The genomic DNA and plasmid DNA containing the insert were amplified using these primers to ascertain the presence of these fragments in the genome and plasmid respectively (Table 18). All the 11 clones have clearly shown the presence of insert both in plasimd and genome (Fig. 28a, b).

4.7.5: Bioinformatic analysis

Bioinformatics analysis was carried out in several steps. In the most favorable cases, the clone gave a "significant" hit by BLASTX with databases of the National Center for Biotechnology Information (NCBI) protein sequences. Clone 1B8 showed a significant hit with BLASTX to a bZIP family transcriptional factor protein of *Arabidopsis thaliana*, Gene Bank accession number AAU94427, e-value 4e-32 (Table 15). Translation of 1B8 clone sequence was done by Edit Sequence Programme of DNA STAR 4.05 and submitted to the translated amino acid sequence to various progamme available on NET for analysis.

First of all, the amino acid sequence was submitted to protein colourer tool of EMBL-EBI (http://www.expasy.org/tools/protparam.html) for amino acid color. The

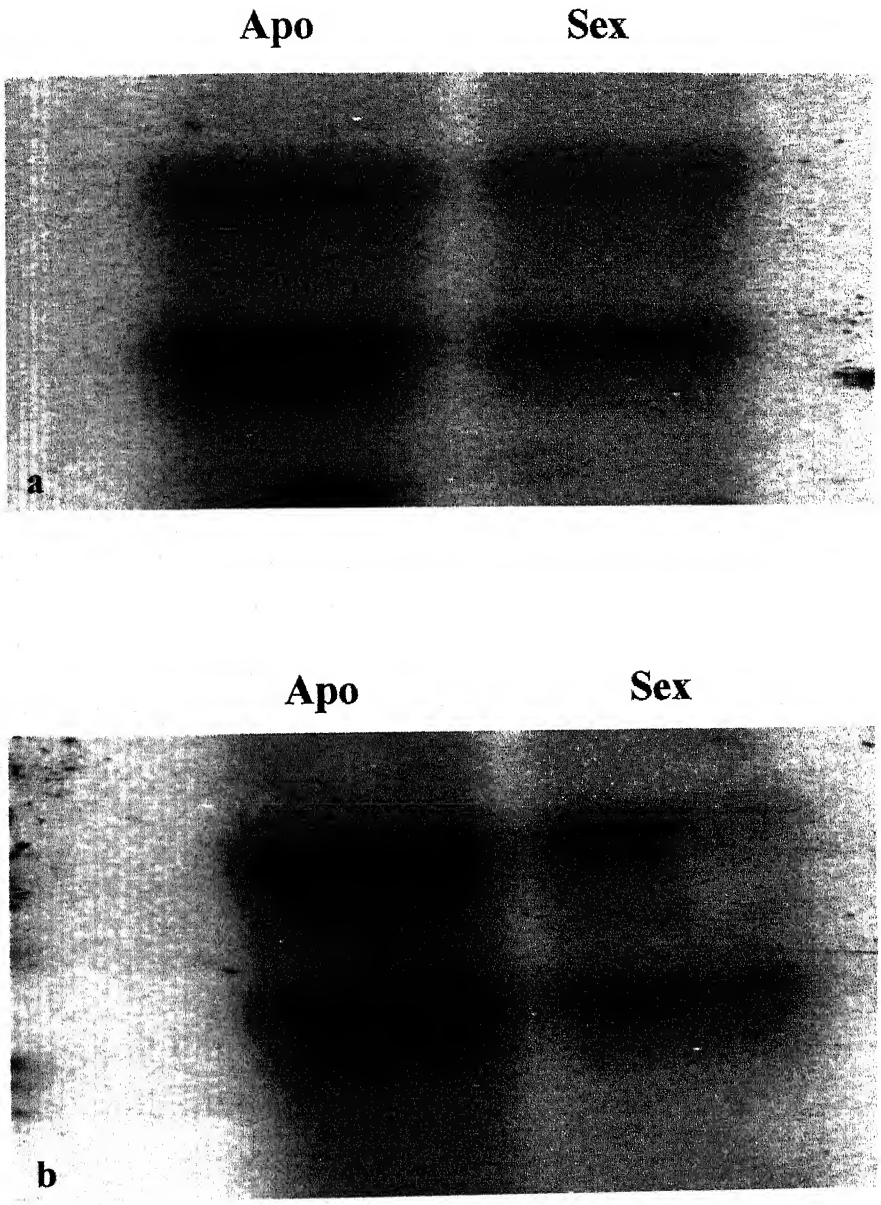
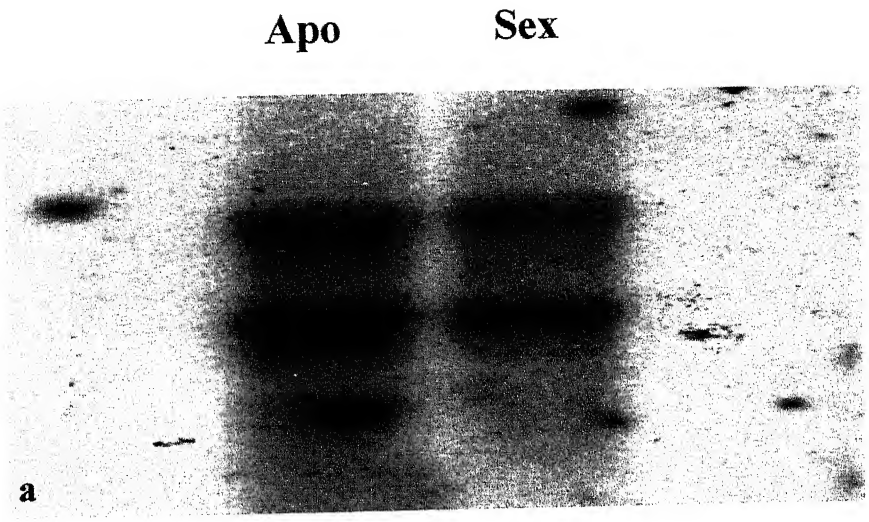


Fig.24: Northern analysis of clones isolated from subtractive hybridization showing expression profiles.
Apo: apomictic plant
Sex: sexual plant
Fig.a: probed with 1A4 clone
Fig.b: probed with 2A6 clone



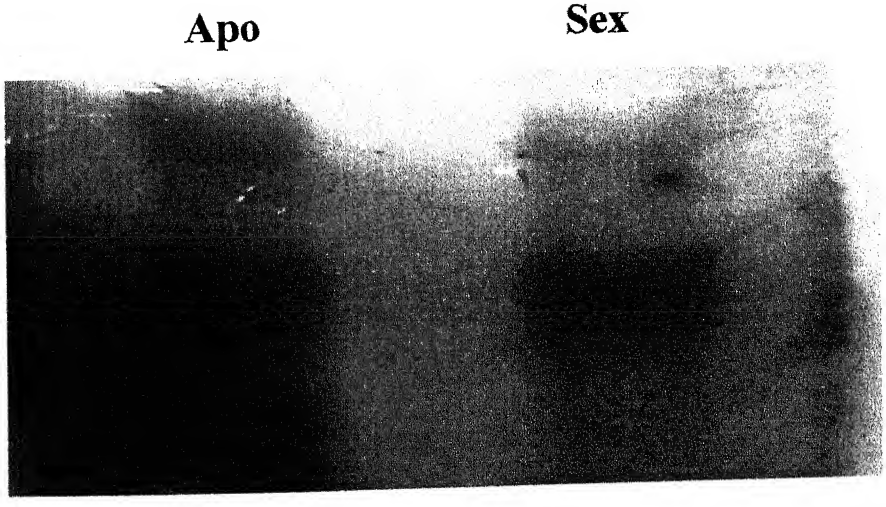


Fig.25: Northern analysis of clones isolated from subtractive hybridization showing expression profiles.
Apo: apomictic plant
Sex: sexual plant
Fig.a: probed with 1G1clone
Fig.b: probed with 2H2 clone

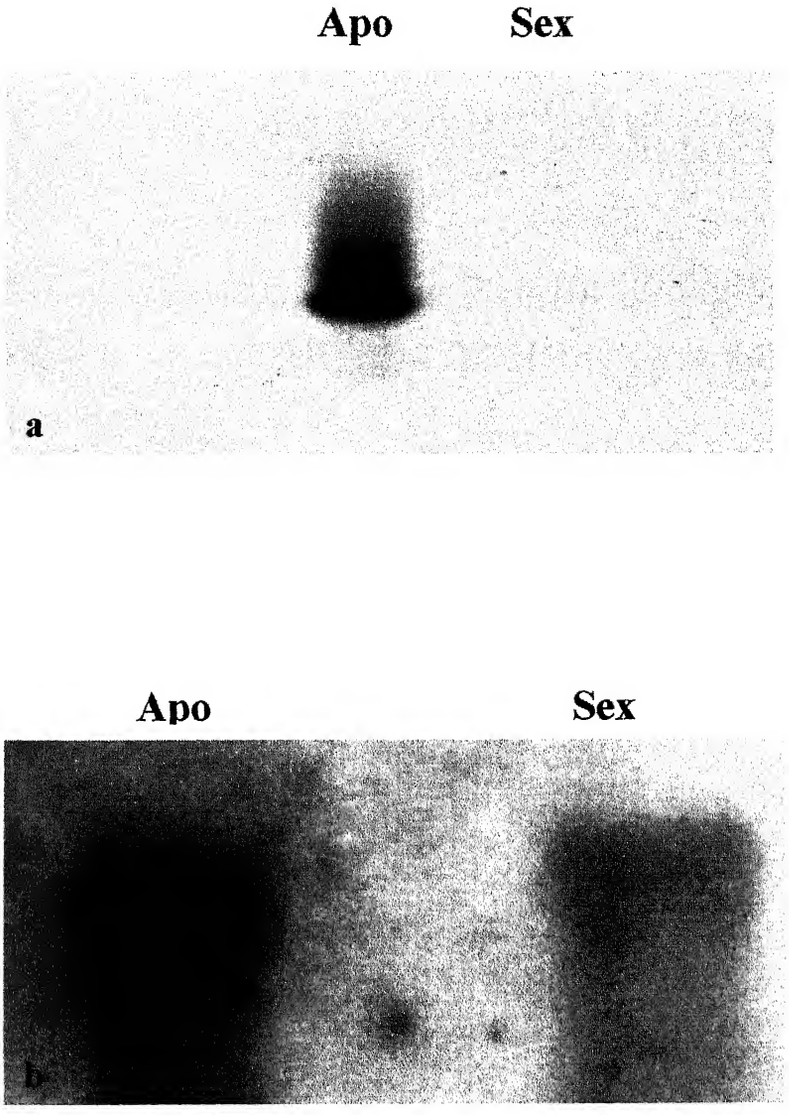


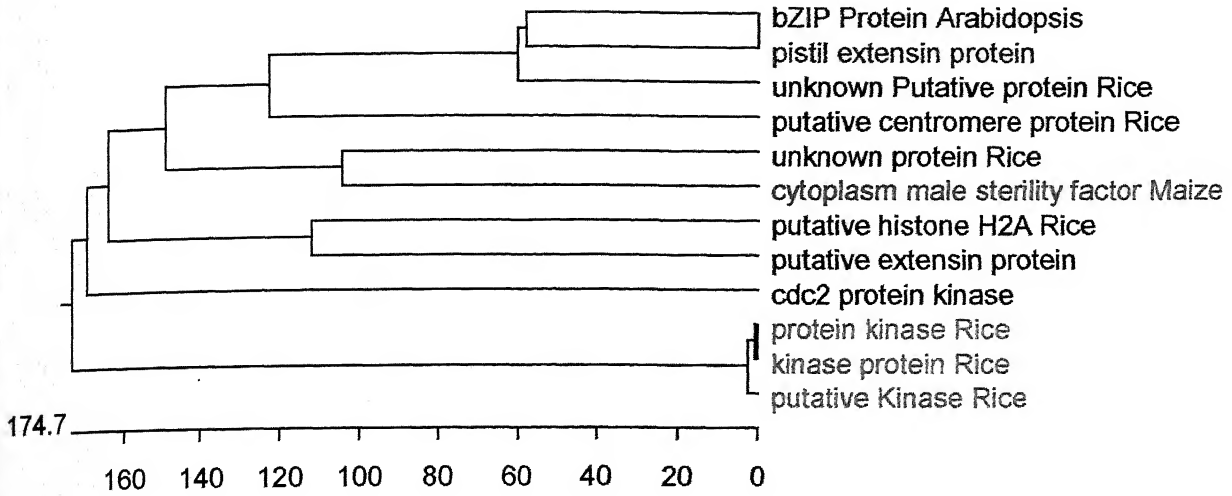
Fig.26: Northern analysis of clones isolated from subtractive hybridization showing expression profiles.
Apo: apomictic plant
Sex: sexual plant
Fig.a: probed with 1B8 clone
Fig.b: probed with 26srRNA (positive control)

Table17. Pattern of expression of promising cDNA clones in the ovaries of apomictic (7-18) and sexual (7-4) F2 segregants as indicated during northern analysis

Clone name used as probe	Expression in RNA blot
1A4, 2A6, 1G1, 2H2	Both types of ovaries (apomictic & sexual)
1B8	Only in apomictic
1C6, 1H8, 1F12, 1E12, 1E3, 2F4, 1F1, 1G4, 1B2, 1H6, 2C4	No signal

Table 18. Characterization of selected cDNA clones by PCR amplification of recombinant plasmid

S.N.	Contig. No.	Clones No.	Size (bp)
1	1	2F10	321
2	6	1E3	756
3	10	1E12	439
4	11	1F1	777
5	15	1F12	591
6	21	1B8	900
7	22	1H8	373
8	25	1A4	600
9	35	2H2	420
10	44	1E9, 2A6, 1G1	571
11	46	1C10, 1C6	200



Clone name	Accession number	Clone identity
1B8	NP_176108	bZIP protein *Arabidopsis thaliana*
2F4	AAK61381	Pistil extensin like protein *Oryza sativa*
1H8	AAT93853	Unknown putative protein *Oryza sativa*
1F1	NP_921528	Putative centromere protein *Oryza sativa*
1E3	AAU92216	Putative unknown protein *Oryza sativa*
1E12	XP_469689	Putative histone H2A protein *Oryza sativa*
1F12	AAP44746	Putative extensin (hypothetical) protein
2H2	AAO00925	cdc2 like protein kinase *Arabidopsis thaliana*
2A6	CAD41324	Putative protein kinase *Oryza sativa*
1G1	AAD54126	Receptor-like protein kinase *Oryza sativa*
1A4	BAB02861	Putative receptor-like protein kinase *Oryza sativa*
Apo-F8	AF215823	Cytoplasm male sterility factor *Zea mays*

Fig. 27: Phylogenetic tree of 11 selected differentially expressed clones with ApoF8 SCAR marker. Using Clustal Mega Align programme of DNASTAR 4.05.

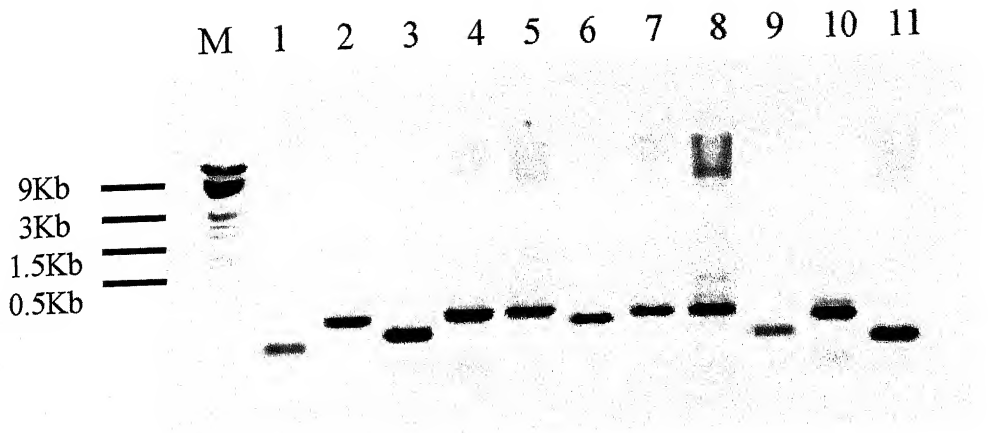


Fig. 28a: PCR amplification of 11selected positive clones by forward and reverse primers of these clones. Plasmid DNA was used as template DNA. M: Lambda DNA/EcoRI+HindIII marker.

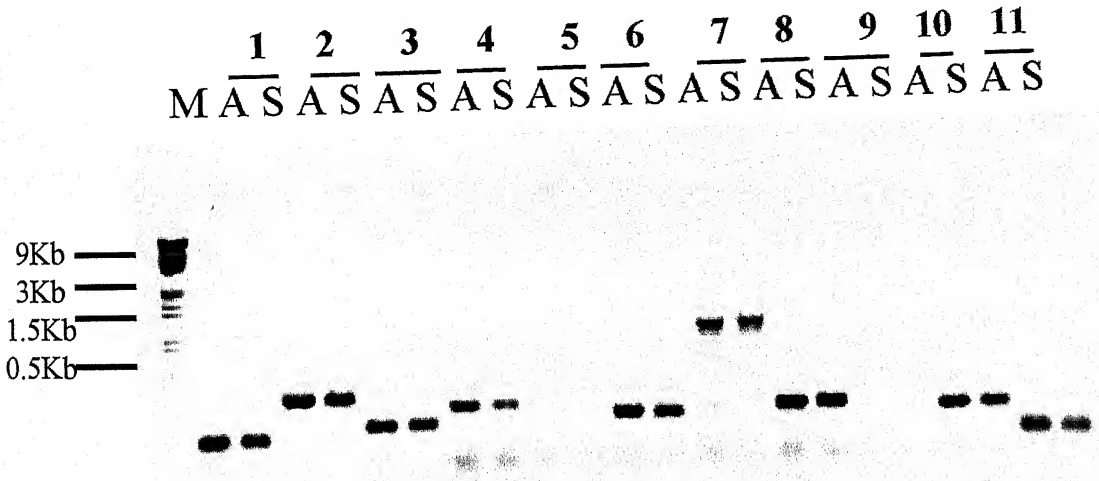


Fig.28b: PCR amplification of total genomic DNA of apomictic (718) and sexual (74) by forward and reverse primers of these clones. A: apomictic, S: sexual, 1 to 11 represents the clones no. M: Lambda DNA/EcoRI+HindIII marker.

amino acids AGZLPV are shown in blue, FYW in red, DENQRHSTK in green and CM in yellow (Fig. 29). In order to search for conserved domain of 1B8 clone, the amino acid sequence was submitted to the NCBI conserved domain finder (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). The CDD results contained a bZIP-conserved domain. The length of the bZIP domain is 21 amino acid long (69-90th residue) this domain is related to Pfam entry 00170; the domain structure is shown in Fig. 30. Using the PROSITE pattern database of expert protein analysis system (http://www.expasy.org/cgi-bin/prosite/scanview.cgi) the sequence of 1B8 clone was scanned and various sites present in sequence were recorded (Post-transnational modification sites). The sequence shows one Glutamine-rich region, one cAMP and cGMP-dependent protein kinase site, one amidation site, two N-myristoylation site, one Leucine Zipper pattern, two N, glycosylation site, nine protein kinase C phosphorylation site and two casein kinaseII phosphorylation site (Fig. 31). Secondary structure prediction was done by using SOPMA (significant improvement in protein secondary structure predication by consensus prediction from multiple alignments) software of NPS (network protein analysis) (Fig. 32).

Phylogenetic analysis of 1B8 clone with other known bZIP family transcription factor was done using the Megalign programme of DNASTAR 4.05. 1B8 clone showed maximum similarity with putative bZIP transcription factor of *Oryza sativa* (Accession no. BAD27924). Whereas it is very distant from bZIP transcription factor RF2B like *Oryza sativa* (Accession no. BAD73033) (Fig. 33).

```
ICHIMYLYLI  IMRRSSPSSS  LVKTLLACVQ  KKKKKKACGR
KLQYIAELEG  RVQALQSEGV  GVSAEMEFLT  QQNIILDLEN
KALKQRLESL  AQEQLIKRFQ  QEMFEREIGR  LRSLYQQQQQ
QQQQQQQQQS  HVLVRSNSRD  LDAQLANLSL  KHKDPNSGRD
ALSGSLRTLL  SSFRSCGFCI  SCHHCVAVFS  IKKGEVRLDC
VNNTGKCLWI  SLFFRDMHLA  VILSLRPLLM  LDQSAVVKGV
LFLVIDAFCA  SLYHYACGSN  LHALPYQWNV  EHSASNKLLE
KCATSLINVV  VLLFISTQAS  MKEVLAFFRV  QVVLDVANLL
KSYQLHNHFN  L
```

Total length: 331

Color code:

Blue : AGILPV
Red : FYW
Green : DENQRHSTK
Yellow :

Fig. 29: Amino acid sequence deduced from nucleotide sequence of 1B8 clone. Using protein colourer tool of Europra Bioinformatics Institute (EMBL-EBI) the above coloration of amino acid was done.

(http://www.expasy.org/tools/protparam.html)

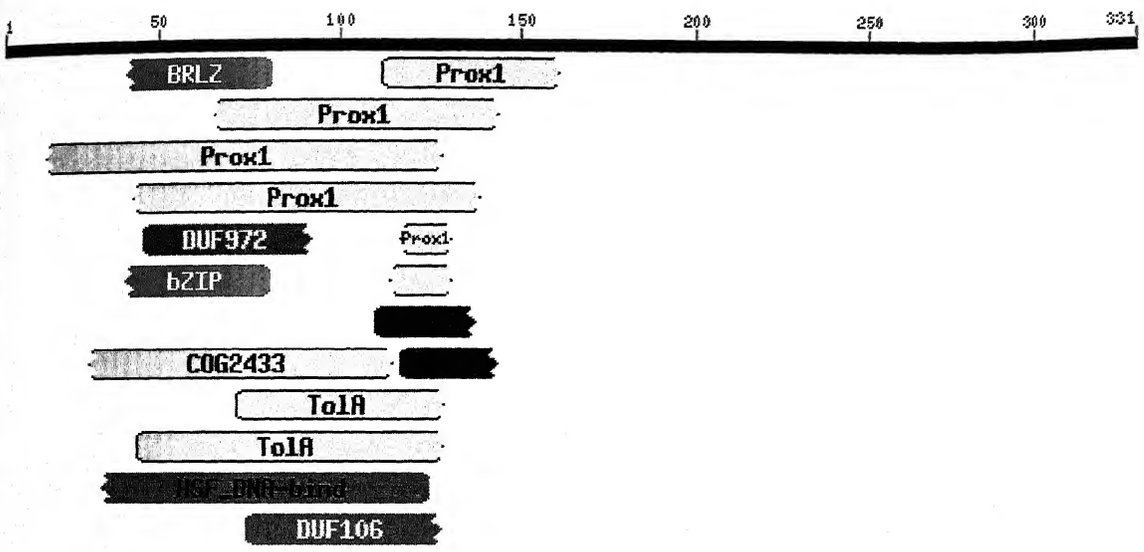


Fig. 30: The conserved domain of 1B8. Using the NCBI conserved domain finder (NCBI conserved domain finder). The protein was found to be of bZIP family. The length of bZIP domain is 21 amino acid (69-90th residue)

(http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi).

Scan Prosite Results Viewer

Found: 19 hits in 1 sequence

hits by profiles with a high probability of occurrence: [1 hit (by 1 profile) on 1 sequence]

Hits by PS50322 **GLN_RICH** *Glutamine-rich region profile* :

92 - 129: **score =** 12.862

QeqlikrfqqemfereigrlrslyqqqqqqqqqqqqqQ

hits by patterns with a high probability of occurrence or by user-defined patterns:

PS00004 **CAMP_PHOSPHO_SITE** *cAMP- and cGMP-dependent protein kinase phosphorylation site* :

13 - 16: RRsS

PS00009 **AMIDATION** *Amidation site* :

38 - 41: cGRK

PS00008 **MYRISTYL** *N-myristoylation site* :

59 - 64: GVgvSA
164 - 169: GSlrTL

PS00029 **LEUCINE_ZIPPER** *Leucine zipper pattern* :

69 - 90: LtqqniiLdlenkaLkqrlesL

PS00001 **ASN_GLYCOSYLATION** *N-glycosylation site* :

147 - 150: NLSL
202 - 205: NNTG

PS00005 **PKC_PHOSPHO_SITE** *Protein kinase C phosphorylation site* :

149 - 151: SlK
157 - 159: SgR

165 - 167: SlR
172 - 174: SfR
190 - 192: SiK
204 - 206: TgK
224 - 226: SlR
275 - 277: SnK
300 - 302: SmK

PS00006 **CK2_PHOSPHO_SITE** *Casein kinase II phosphorylation site* :

157 - 160: SgrD
300 - 303: SmkE

Fig. 31: Post-translational modification sites present in 1B8. Using the PROSITE pattern database of expert protein analysis system the sequence of 1B8 clone was scanned and various sites present in sequence were recorded The sequence shows one Glutamine-rich region, one cAMP and cGMP-dependent protein kinase site, one amidation site, two N-myristoylation site, one Leucine Zipper pattern, two N, glycosylation site, nine protein kinase C phosphorylation site and two casein kinaseII phosphorylation site.

(http://www.expasy.org/cgi-bin/prosite/scanview.cgi)

SOPMA result for : 1B8xxx0

Abstract Geourjon, C. & Deléage, G., SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments., Cabios (1995) 11, 681-684

View SOPMA in: [MPSA (Mac, UNIX) , About...] [AnTheProt (PC) , Download...] [HELP]

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
ICHIMYLYLIIMRRSSPSSSLVKTLLACVQKKKKKKACGRKLQYIAELEGRVQALQSEGVGVSAEMEFLT
hhhhhhheeeee      hhhhhhhhhhhttt    tt hhhhhhhhhhhhhhhhhtt  ehhhhhhhh
QQNIILDLENKALKQRLESLAQEQLIKRFQQEMFEREIGRLRSLYQQQQQQQQQQQQQQSHVLVRSNSRD
ttheeehhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh     eeeee  hh
LDAQLANLSLKHKDPNSGRDALSGSLRTLLSSFRSCGFCISCHHCVAVFSIKKGEVRLDCVNNTGKCLWI
hhhhhhhhhe         hhhhhhhhhhhhhhhttt ee    eeeeeeettt eeeeee tt  eeee
SLFFRDMHLAVILSLRPLLMLDQSAVVKGVLFLVIDAFCASLYHYACGSNLHALPYQWNVEHSASNKLLE
eeehhhhhhhhhhhhhhheee thhhhhteeeeeehhhhhhhhhh tt  ee  eeeee hhhhhhhhh
KCATSLINVVVLLFISTQASMKEVLAFFRVQVVLDVANLLKSYQLHNHFNL
hhhhhhheeeeeee hhhhhhhhhhhhhheehhhhhhhhht    e
```

```
Sequence length :   331

SOPMA :
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   3_10 helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    63 is  19.03%
   Beta turn        (Tt) :    22 is   6.65%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    58 is  17.52%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%


Parameters :
   Window width          :  17
   Similarity threshold  :   8
   Number of states      :   4
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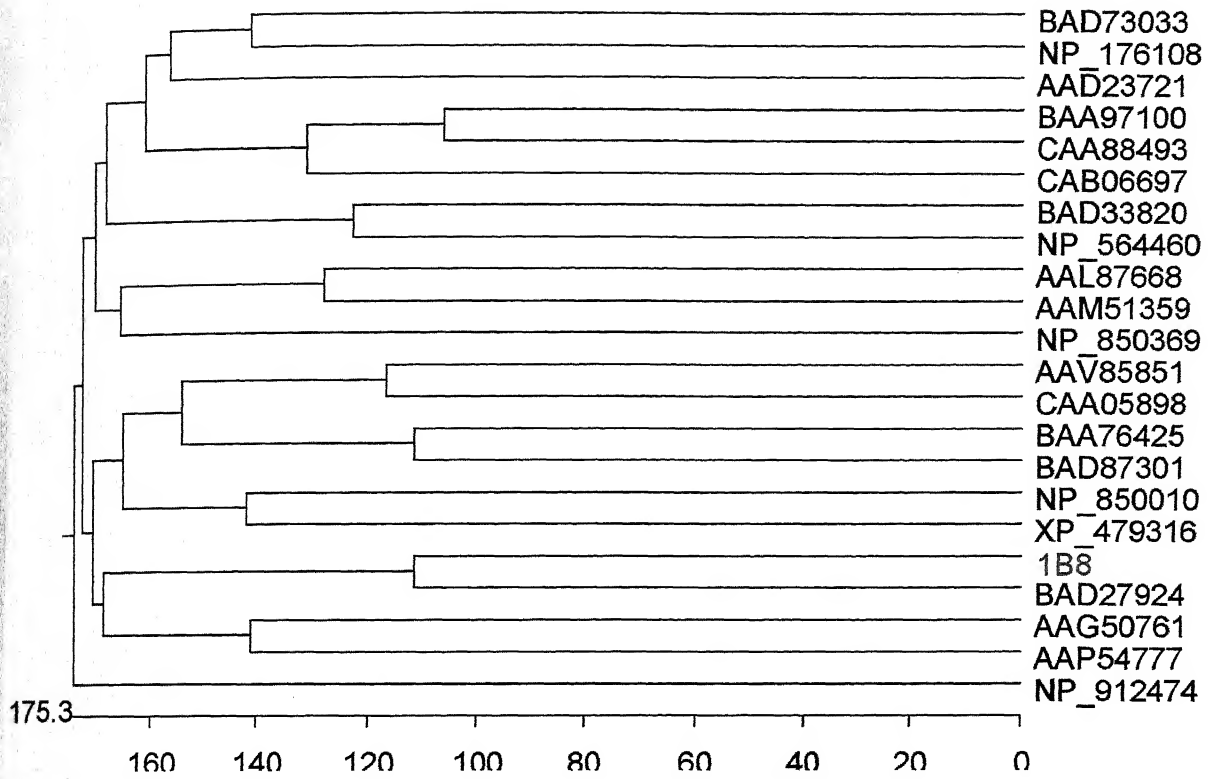
Prediction result file (text): [SOPMA]
Intermediate result file (text): [BLASTP on SWISS-PROT]

Fig. 32: Secondary structure prediction was done by using SOPMA (significant improvement in protein secondary structure predication by consensus prediction from multiple alignments) software of NPS (network protein analysis).



Accession number	Clone identity
1. NP_176108	*Arabidopsis thalina* bZIP transcription factor
2. AAG50761	*Arabidopsis thalina* hypothetical protein
3. BAD87301	Putative transcription activator RF2a *Oryza sativa*
4. NP_564460	bZIP family transcriptional factor *Arabidopsis thalina*
5. NP_850369	bZIP transcription factor *Arabidopsis thalina*
6. AAD23721	*Arabidopsis thalina* expressed protein
7. BAD73033	bZIP transcription factor RF2B like *Oryza sativa*
8. BAD27924	Putative bZIP transcription factor *Oryza sativa*
9. NP_912474	Putative bZIP transcription factor *Oryza sativa*
10. BAA97100	bZIP transcriptional activator RSG *Nicotiana tabacum*
11. BAD33820	bZIP transcription factor RF2a like *Oryza sativa*
12. AAP54777	Putative bZIP transcription factor *Oryza sativa*
13. CAB06697	bZIP DNA binding protein *Arabidopsis thalina*
14. BAA76425	bZIP DNA binding protein *Cicer arietinum* (Chickpea)
15. CAA05898	Transcriptional factor VSF-1 *Lycopersicon esculentum*
16. AAL87668	Susceptibility transcription factor RVS1 *Oryza sativa*
17. NP_850010	bZIP protein related *Arabidopsis thalina*
18. AAM51359	Putative VirE2 interacting protein VIP1 *Arabidopsis thalina*
19. XP_479316	Putative bZIP family transcription factor *Oryza sativa*
20. CAA88493	TAF-3 (TAF family of tobacco) G-box binding proteins
21. AAV85851	AT-rich element binding factor1 *Pisum sativum*

Fig. 33: Phylogenetic tree of 1B8 clone with other known bZIP family genes, Using Clustal Mega Align programme of DNASTAR 4.05.

DISCUSSION

CHAPTER 5
DISCUSSION

Cloning of genes for apomixis from apomictic species and their introduction into sexual species through transformation is an exciting possibility. Two methods have been proposed for the isolation of such genes: map based cloning and subtractive hybridization. If the genes for apomixis can be tagged with molecular marker such as RAPDs, breeding material can be screened for apomixis through linked markers and this marker can also be used to map the locus controlling apospory, which can then be cloned by chromosome walking.

The first and most comprehensive mapping efforts in apomicts have been reported from groups working in grass species, most of which are relatives of important cereal crops. The dominant factor associated with the inheritance of apomixis in the aposporous plus pseudogamous grass species *Pennisetum squamulatum* appears to be hemizygous, because no equivalent region has been found in sexual biotypes (Ozias-Akins *et al.*, 1993, 1998; Roche *et al.*, 2001a). Intriguingly, the same region was found in the apomictic relative *Cenchrus ciliaris*, also as a hemizygous region (Roche *et al.*, 1999, 2001a). Efforts to isolate this region have revealed its complex nature. Twelve molecular markers were reported to cosegregate with the apospory-specific genomic region without separation, an early indication that this region may be associated with the localized suppression of recombination. After the isolation of BACs corresponding to these markers, it became apparent that this region of suppressed recombination spanned at least 50Mbp in *P.squamulatum* (Roche *et al.*, 2002) and may be considerably larger. Using the corresponding BAC clones as probes, Goel *et al.* (2003) demonstrated that the apospory-specific genomic region localize to a single short arm of *P.squamulatum* chromosome.

Subtractive hybridization method involves subtraction of mRNA population of apomictically reproducing ovaries from the sexual reproducing ovaries. The mRNA species, which are not hybridized, are cloned into a cloning vector for sequencing and characterization. In the present study a mapping population which cosegregated with apomixis and sexuality was used to isolate the gene associated with apomixis while the

suggestive markers that co-inherited for apomixis and sexuality were used to fingerprint these genotypes. Using an advanced method such as subtractive hybridization, many partial cDNA fragments have been cloned and sequenced.

5.1 Mapping population

A proper mapping population that cosegregated with the desired trait is a prerequisite for any genetic and molecular studies. In the present study, a single obligate sexual plant was left to open pollination for the development of halfsibs. On screening they showed two different modes of reproduction, obligate apomicts and facultative apomicts. Three facultative halfsibs were further selfed to develop F2 segregants, which showed obligate sexual, obligate apomictic and facultative apomictic types. The pattern of segregation indicated apomixis as dominant mode of inheritance. But the genetics of inheritance of apomixis could not be analyzed due to the unknown pollen donor. This is the first such mapping population used for the study of apomixis. The earlier studies either used F1 population (Ozias-Akins *et al.*, 1998; Albertini *et al.*, 2001a; Jessup *et al.*, 2002; Martinez *et al.*, 2003) or the half sibs (Gustine *et al.*, 1996). For cloning the genes for apomixis the F2 population used in the present study had more advantages, since false positives being cloned which are not associated with apomixis would have been very low.

5.2 Embryo sac analysis

In the present study embryo sac analysis was carried out using ovule clearing technique of Young *et al.*, (1979). Three kinds of ovules observed in the present study were also reported in several other studies. In most aposporous ovules the meiotically derived megaspores abort, and the differentiation of a somatic unreduced nucellar cell gives rise to a four nucleated embryo sac (Gustafsson, 1947a) which contains the equivalent of two synergids, an egg cell and a polar nucleus (Nogler, 1984a). While this pattern of differentiation may vary, antipodals normally are not present at the chalazal end of the central cell. In most cases, the fusion of sperm and egg cell is prevented, but fertilization of the polar nucleus is often required for the production of viable seed (Vielle *et al.*, 1995).

Clearing technique using non-aqueous fluids (Herr, 1971; Young *et al.*, 1979; Crane and Carman, 1987) now represent the best tool for observing ovule details during both megasporogenesis and megagametogensis in aposporous and diplosporous materials. Procedure combining Mayers hemalum staining with methyl-salicilate clearing have been successfully used for observations within whole ovules of *Solanum* (Stelly *et al.*, 1984) and *Medicago* (Tavoletti *et al.*, 1991). These techniques are of great interest for embryological analyses in apomicts because they do not require the use of special optics.

In *C. ciliaris*, as in most reported aposporous species, female gametophytes usually contain two synergids, an egg cell, and a single polar nucleus in the central cell; however, a variable number of female gametophytes (up to 20% in certain genotypes) may be composed of a single synergid, an egg cell, and two polar nuclei. On rare occasions, embryo sacs containing three polar nuclei and no synergids have been observed. This variable organization appears to be associated with the localization of nuclei prior to cellularization, and suggests the positional information plays a role in gametophytic cell specification (Savidan *et al.*, 2001).

5.3 Protein polymorphism

Our results have indicated stage specific proteins that are expressed during apomictic and sexual pathways. 97KDa protein was associated with only pre meiotic sexual, while 80KDa protein complement was absent only in apomictic post meiotic ovaries. A 60KDa protein was observed in sexual ovaries undergoing both pre meiotic and post meiotic stages, it was conspicuously absent in both the stages of apomictic ovaries. Another protein of Mol. Wt. 50KDa was associated with pre meiotic ovaries of both the sexual and apomictic plants, while it was absent in post meiotic ovules of both the plants. A total of 17 polypeptides were recorded. The earlier studies on this grass could not separate any steady state proteins of pistils at either pre meiotic or post meiotic stages, although 308 spots showing nearly 12% polymorphism have been observed (Gustine *et al.*, 1996). But none of the proteins were found to co-segregate with the reproductive mode. Whereas Gounaris *et al.*, (1991a), had earlier reported 4 stamen specific proteins out of 308 spots examined. One of them was specific to microsporogensis stage and another to a uninucleate microspore stage and other two were found in both the stages of megasporogenesis and microsporogensis in *Cenchrus ciliaris*.

There was a great similarity between the stamen and pistil protein complements, where only four proteins were unique to stamen and none to pistils. This similarity could be due to the material used for the study i.e. halfsib population. Similarly Koul and Bhargava (1986), found a few anther specific isoperoxidase during sporogenesis.

Our study also indicated that there might be genes regulating these processes may have protein products, which are detectable. To associate these proteins with meiosis specific or gametophyte specific stages required further characterization. While the above referred earlier studies were conducted on half-sib progenies of sexual and apomictic parental material, our study was conducted among the F2 segregants of half-sibs which were more genetically related. This has further reduced the chances of occurrence of false positives that are unrelated to reproductive development.

Stage specific proteins have also been detected in the reproductive tissues of the early stages of formation of sporogeneous tissues (Kahlem, 1976) or the microspores (Singh *et al.*, 1985). Proteins that are both organ specific and stage specific are interesting because their correlation with form and function indicated possible role for the proteins. All the four proteins in our study differentiated specifically with either pre or post-meiotic apomictic or sexual ovules. They need to be further characterized and studied in detail. Results of study by Vielle *et al.*, (1995) also corroborated our findings clearly indicating differences between the early fertilization events in sexual and apomictic female gametophytes of *Cenchrus ciliaris*. They compared sexual and aposporous egg apparatus (synergids and egg cell) of both sexual and apomictic F1 segregants unpollinated and pollinated pistils prior to the pollen tube entry into the female gametophyte.

In an another recent study (Agafonov *et al.*, 2004), on identification of apomixis in the Kentucky blue grass using SDS-PAGE of endosperm storage proteins, it was difficult to distinguish hybrids and autonomous endosperms in the case of meiotic forms by the results of SDS-PAGE. Variability in protein composition may be observed in the case of both sexual origin of endosperm (i.e., outbreeding or inbreeding of heterozygotic genotype) and apomixis (pseudogamous and autonomous), if meiotic processes occurred during megasporogensis. They opined that the endosperm development as a result of inbreeding or apomixis may be identified by the combination of protein markers of the maternal and pollinator genotypes in the endosperm.

5.4 Isozyme polymorphism

Analysis of isozymes of six enzymes namely Esterase, Acid phosphatase, Aspartate amino transferase, Phosphoglucomutase, Glucose-6-phosphate dehydrogenase and Superoxide dismutase has revealed polymorphism among ovaries of sexual and apomictic F2 segregants. There was polymorphism between pre meiotic and post meiotic stages of both the types. Out of 43 putative alleles scored among 14 loci, 37 alleles were recorded in the pre meiotic stages while only 24 alleles were recorded for post meiotic stage. Maximum polymorphism of Esterase isozyme with all the 6 alleles of Est-1 associated with pre meiotic stages clearly indicated their stage specific expression. 3 alleles of Est-1 were conspicuously absent in the ovaries of sexual plant, but 3 loci, out of 14 loci namely ACP-1, ACP-2 and AAT-2 did not show polymorphism. This is the first report on differentiating isozyme during pre and post meiotic gametogenesis.

Out of 6 enzymes analyzed maximum polymorphism was observed for Esterase and Phosphoglucomutase, while Glucose-6 phosphate dehydrogenase and Superoxide Dismutase did not show significant polymorphism. This could be due to the type of material used, as they were genetically more homogenous. Moreover the genes encoding these enzymes may be conserved. On the contrary, Gustine *et al.*, (1996) observed polymorphism in 12 out of 22 isozyme systems tested using leaf extracts where none of the isozyme co-segregated with apomixis. Higher polymorphism in their study could be due to the use of halfsib families, segregating for the mode of reproduction. Although halfsib families showed more polymorphism the probability of identifying a cosegregating marker for apomixis is higher when F2 segregants for the mode of reproduction are used. However, our study did not include segregation analysis for protein and isozyme, due to unknown paternal parent used, but this is the first study conducted using ovaries instead of florets. Moreover, previous studies for isozymes used low resolving starch gel electrophoresis system compared to native PAGE used in our study.

In an another study, isozyme polymorphism of the sexual pool was higher (69%) compared to apomictic pool (Assienan and Noirot, 1995). Overall polymorphism was considerable indicating that apomixis does not lead to a reduction in diversity. The existence of three to four alleles at many loci (EST and ACP) leads us to assume *a priori*

the presence of a high level of heterozygosity in apomicts by the maintenance of quadruplex or triplex structures. Similar observation was made by Assienan and Noirot (1995), who did not observe any quadruplex type. This situation clearly showed that the advantage of the polyploidy-apomixis relationship is not to maintain the within individual heterozygosity level of apomict higher than that of a diploid. In our study, ACP-3, Est-3 and Est-4 indicated specific alleles associated with apomictic ovaries, whereas AAT-1, AAT-2, AAT-3 and G6PDH-1 loci exhibited alleles specific to sexual ovaries. In a survey of 50 enzymes of *Hordeum vulgare*, Pedersen *et al.*, (1987) noted six anther specific enzymes. Koul and Bhargava (1986) also reported a few anther specific isoperoxidases during sporogenesis. Alcohol dehydrogenase activity first appeared after tetrads began to break a part in *Zea mays* (Stinson and mascarenhas, 1985). None of the studies on isozymes sofar was related to developmental stages of ovules.

Two major groups comprising of pre meiotic apomictic and sexual ovaries and post meiotic apomictic and sexual ovaries in protein based dendrogram in our study clearly indicated a definite pattern of stage specificity of protein. The differentiation of proteins was based on pre meiotic and post meiotic stages than on the modes of reproduction. This could be due to the specific proteins produced during megasporogensis or megagametogensis. But the dendrogram analysis based on isozyme indicated grouping of pre and post meiotic sexual types including post meiotic apomictic types delineating pre meiotic apomictic type. This clearly implied the possibility of specific isozyme associated with pre meiotic apomictic ovaries and seemed to play a definite role during early megasporogenesis than during the later stages of megasporogenesis and megagametogensis of apomicts. Lack of isozyme polymorphism between pre and post-meiotic sexual ovaries indicated that isozymes are not differentially regulated during these stages, as to be expected. Distinct grouping of pre meiotic apomictic ovaries was due to associated isozyme loci implying a different pattern of gene regulation during premeiotic megasporogenesis.

The difference in the clustering of these stages between protein and isozyme based dendrogram could be due to the nature of inheritance of proteins and isozymes. While isozymes are influenced by the developmental stage, proteins are the products of conserved genes. From this angle, a pattern based on protein might be more genetically based while for that of isozymes it may be due to stage specificity. This is the first ever study conducted to analyze the phylogenetic pattern.

5.5 DNA polymorphism and fingerprinting

DNA based polymorphism analysis reveals a pattern of markers that can uniquely identify an individual. Tracking the inheritance of such markers from parents to offspring is a very precise method of confirming parentage as well as clonal reproduction (Grattapagila *et al,* 1996). DNA amplification by the polymerase chain reaction using single primer of arbitrary sequence has tremendous practical applications. The suitability of arbitrarily primed amplified fragments as genetic markers (Williams *et al,* 1990; Klein-Lankhorst *et al,* 1991), for fingerprinting (Welsh and McClelland, 1990, 1991) and for strain identification and genetic mapping (Welsh *et al.,* 1991) has been established. We have used bulked segregant analysis or BSA (Michelmore *et al.,* 1991) to determine extent of polymorphism, to identify closely linked markers to apomixis and to study population and phylogenetic parameters.

BSA is now commonly performed in molecular genetic study for identification of markers linked to traits of agronomic value and bulked screenings are usually performed in the absence of the linkage map that is valid for any segregating population of the species concerned (Pessino *et al.,* 1997). Two bulks of DNA randomly collected from five apomicts and eight sexual plants were screened in the present study using 208 random decamer for identifying RAPD polymorphism. This screening facilitated rejection of primers which either did not amplify (22.5%) or amplified faintly (12%) or were monomorphic (55.7%). Twenty primers (9.6%) were found to be polymorphic but from these, fourteen primers did not show associated banding patterns among sexual and apomictic types. Only six primers exhibited reproducible polymorphism and had given rise to 36 alleles, which ranged from 300bp to 3Kb. The previously reported markers, OPC04 and UGT197 (Ozias-Akins *et al.,* 1993, Lubbers *et al.,* 1994) did not show polymorphism in our F2 mapping population. Later, Gustine *et al.,* (1997) reported two RAPD markers M2 (680) and J16 (800) tightly linked to apospory using a bulked segregant analysis.

RAPD analysis was successfully used to investigate the relationship between apomictic plant species. In a study on *Taraxacum* (Reisch, 2004) different species relationships were analyzed by RAPD patterns. The size of the amplified fragments ranged from 250bp to 2540bp and the percentage of bands per primer, which were

polymorphic varied between 62.5% and 100%. Over all 78.4% of the amplified bands were polymorphic.

Low level of polymorphism among the primers used for screening the bulks in our study can be attributed to the genetically closer F2 segregants used. In the previous studies either halfsibs or F1s were used in *Cenchrus ciliaris*. But screening of F2 population could help in identifying more closely linked RAPD markers to apo-locus. Another reason for low amount of polymorphism could be the inclusion of less number of individuals in each bulk. 36 alleles from 6 primers were able to clearly discriminate among apomictic and sexual segregants.

Number of electrophoretic phenotypes (EPs) is an another parameter to assess the genetic variation among apomictic and sexual segregants. More number of EPs among sexual segergants over apomictic segregants indicated higher amount of genetic variation among the sexual segergants. This is also reflected in the amount of polymorphism (69.44%), which is higher in sexual segregants compare to apomictic segregants.

All the population genetic parameters such as observed number of alleles per locus, effective number of alleles per locus, gene diversity, Shannon's information index estimated in our study indicated higher amount among the sexual segregants. This could be due to higher amount of genetic recombination in sexual segregants. Moreover, our mapping population consisted of half-sib progenies of three halfsibs. There was a possibility of greater amount of differentiation from the facultative apomictic halfsibs producing heterogeneous sexual F2 segregants than apomictic F2 segregants. Although agamosperms are known to have possible elevated level of heterozygosity in nature, our study has indicated low levels. Observed heterozygosity in various allopolyploid apomicts ranged from 0.128 in *Antennaria parlinii* (Bayer and Crawford, 1986) to 0.684 in *Taraxacum albidum* (Menken and Morita, 1989). Whereas it ranged from 0.063 in *Antennaria monocephala* (Bayer, 1991) to 0.097 in *Antennaria friesiana* (Bayer, 1991). But there was no difference found between agamosperm and sexual plants of *Panicum maximum* (Assienan and Noirot, 1995). The population genetic parameters were estimated for the first time in *Cenchrus ciliaris*, where a higher percent polymorphism was observed in the sexual populations. On the contrary, previous reports have all shown higher percent polymorphism among apomicts than their sexual relatives. As high as 69% polymorphism was recorded in *Panicum maximum* (Assienan and Noirot, 1995). Even mean number of alleles per locus per population reported earlier were higher

ranging from 1.1(Yahara *et al.*, 1991) to 1.8 (Menken and Morita, 1989). Whereas our study has indicated 1.33 and 1.69 for apomictic and sexual respectively.

To identify primer that could distinguish F2 segregants more efficiently polymorphism information content was determined for all the primers. Primer OPF-08 with a value of 1 was found to be the ideal primer for generating polymorphism. Rana and Bhat (2004), has also used this parameter for identifying polymorphic primer. Anderson *et al.*, (1993) used PIC for ranking of clones.

Dendrogram analysis was carried out to analyze the phylogenetic relationship among the material used. Two clear clusters each comprising of apomictic and sexual segregants with respective bulk being included, indicated the high discriminating power of primers used in the study. Although the 13 F2 segregants belonged to 3 half-sibs, they were grouped according to their modes of reproduction indicating the power of resolution of the RAPD for fingerprinting. It was also interesting to note that in majority of the cases the progenies of a single half-sib showing a particular mode of reproduction showed maximum similarity. This was more clearly analyzed by the similarity indices. The highest similarity indices, 0.944 and 0.833 were between apomictic and sexual F2 segregants respectively, which have arisen from two different F1 half-sibs. Moreover similarity indices for apomictic F2 segregants were higher than for sexual segregants. This can be attributed to the apomictic nature of reproduction among apomictic progenies, resulting into more homogenous progenies. The apomictic and sexual bulks have also grouped into two different clusters along with their respective segregants, thus validating our sampling procedure in the study. Dendrogram analysis has also shown the mother plant in the cluster consisting of sexual segregants. Formation of two different clusters between apomictic and sexual individuals has also shown considerable amount of DNA polymorphism for this trait in *Cenchrus ciliaris*. Generally, obligate agamosperms are known to persist without substantial genetic recombination. An extreme example is the complete lack of RAPD polymorphism in the obligate apomict *Limonium cavanillesii* (Palacios and Gonzalez-Candelas, 1997). Nevertheless, genetic variability does exist to some extent in several obligate apomictics (Richards, 1986).

While determining the mode of reproduction in *Paspalum notatum* using both RFLP and RAPD markers, Ortiz *et al.* (1997) have reported genetic fingerprints for studying the level of apomixis. Casa *et al.* (2002), analyzed genetic variability among sexual and apomictic *Paspalum dilatatum* using 362 RAPD fragments, genetic similarity

estimates revealed that the penta and hexaploid biotypes were highly similar ($S_D \geq 0.913$). Forty RAPD markers were unique to the penta and hexapliod biotypes. Overall, RAPD markers were useful for assessing genetic variation among closely related *P. dilatatum* genotypes as well as generating putative X genome markers.

5.6 Development of SCAR marker

A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucletide primers. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into codominant markers. SCARs are similar to the sequence-tagged site (STSs) that have been proposed by Olson *et al.*, (1989), as DNA land-marks in the physical map of the human genome. SCARs are primarily defined genetically; therefore, they can be used not only as physical landmarks in the genome but also as genetic markers. In addition, SCARs can contain repetitive DNA sequences within amplified fragment as they are analyzed by PCR only; their uniqueness is determined by sequence and spacing of the primer sequences, rather than by hybridization (Paran *et al.*, 1993).

We derived one SCAR marker by cloning an amplified product of RAPD marker OPF-08 and sequencing from both the ends of the marker. The 600 bp sequence was used to design pairs of 23 and 24 mers oligonucleotide primers that resulted in reproducible amplification of single locus, when high annealing temperature was used. This marker was closely associated with apomictic F2 segregants, while it was absent in the sexual F2 segregants. Bulked segregant analysis has been successfully used in the identification of markers linked to the apomictic reproduction in other grasses like *Tripsacum* (Leblanc *et al.*, 1995), *Brachiaria* (Pessino *et al.*, 1997 and 1998) *Pennisetum ciliare* (Gustine *et al.*, 1997), *Poa* (Barcaccia *et al.*, 1998) *and Paspalum* (Martinez *et al.*, 2003). In the present study segregation analysis could not be done due to unknown paternal parent. Since the F2 segregants belonged to three half-sibs, OPF-08 SCAR marker is considered as a highly reproducible and closely linked to apomixis. Hence, this SCAR marker could be used in the DNA fingerprinting of apomictic genotypes of *C. ciliaris*. In *Pennisetum*, Ozias-Akins *et al.*, (1998) found 12 molecular markers (11 SCARs and 1 STS) showing complete linkage to the apospory locus in a progeny of 397 individuals that segregated for apospory in a Mendelian 1:1 ratio. A subset of these

markers were also linked to apospory and showed no recombination in *Cenchrus ciliaris* (Roche *et al.*, 1999). In *Cenchrus ciliaris* (Roche *et al.*, 1999), *Pennisetum squamulatum* (Ozias-Akins *et al.*, 1998) *and Tripsacum* (Grimanelli *et al.*, 1998b), lack of recombination around the apomixis locus was also observed. The possible causes for this lack of recombination around the apospory locus have been discussed by Ozias-Akins *et al.*, (1998). Among the different explanations proposed, they hypothesized that the apospory locus is situated in chromosome positions in which the recombination is known to be repressed such as pericentromeric or other heterochromatic regions, or even in an unpaired minichromosome. In other species like *Brachiaria*, by means of comparative mapping with maize, it was suggested that the apospory locus could be located in the telomeric region (Pessino *et al.*, 1998). Another possible explanation could be the localization of the apospory locus in a heterozygous inversion or in an introduced region of DNA from another species (Ozias-Akins *et al.*, 1998; Pupilli *et al.*, 2001). The third possibility is that the specific region of the apospory is a complex locus controlled by two or more genes, which are maintained as a unit, and where recombination is suppressed by some of the mechanisms mentioned previously (Ozias-Akins *et al.*, 1998). Savidan (2000a) proposed the existence of a linkat, as a hypothesis to explain the suppression of recombination in the apomixis locus. A linkat was defined by Demarly (1979) as a segment of chromosome in which the linkage of genes contributing to a single function are tightly kept together, e.g. by means of inversion.

SCAR marker OPF-08 was further characterized by southern analysis. While there was no hybridization signal with the DNA of sexual individuals, two bands were observed in all the apomictic segregants. When these two bands were eluted and amplified by using SCAR primers, 600 bp fragments were observed in both the cases, implying that their might be two loci for this marker. This could be due to the segmental allopolyploidy nature of *Cenchrus ciliaris* (Jessup *et al.*, 2003). SCAR markers such as Parth1 and Sex1 were also used to detect mode of reproduction in a wide range of facultative apomictic Kentucky blue grass varieties (Albertini *et al.*, 2001a).

5.7 Subtractive hybridization

The isolation of genes associated with apomixis would improve understanding of the molecular mechanism of this mode of reproduction in plants as well as open the possibility of transfer of apomixis to sexual plants, enabling cloning of crops through seeds. To gain an insight into the molecular basis of apomictic reproduction, gene expression in mature ovaries containing reduced and unreduced embryo sacs was compared in *Cenchrus*, an apomictic tropical forage grass. In this study we have compared gene expression in ovaries at megasporogenesis and megagametogenesis of sexual and apomictic accessions of *C. ciliaris* by PCR based subtractive hybridization (Reddy *et al.*, 2002).

A total of 141 cDNA clones were cloned into pBluescript KS+ cloning vector, out of which 70 clones were finally selected after sequence analysis. These clones have shown homology to various genes and proteins. Based on the possible relationship of these genes with the reproductive mechanism, 16 clones were further analyzed by reverse northern analysis. One clone namely 1B8 of size 1029bp showing homology to bZIP family transcriptional factors has been identified as a possible gene associated with apomixis. In plants, basic region/leucine zipper motif (bZIP) transcription factors regulate processes including pathogen defence, light and stress signaling, seed maturation and flower development. The *Arabidopsis* genome sequence contains 75 distinct members of the bZIP family, of which ~50 are not described in the literature (Jakoby *et al.*, 2002).

Earlier, differentially expressed mRNAs were identified in *Pennisetum* (Vielle-Calzada *et al.*, 1996b); *Brachiaria* (Leblanc *et al.*, 1997); *Panicum* (Chen *et al.*, 1999) *and Paspalum* (Pessino *et al.*, 2001), but none of them was characterized as responsible for the apomictic trait. Recently Julio *et al.*, (2003), reported specificity of 65 cloned fragments, checked by reverse northern blot analysis, showed that 11 clones were differentially expressed, 6 in apomictic ovaries, 2 in sexual and 3 in apomictic and sexual, but at different stages. Of 6 sequences isolated that were preferentially expressed in the apomictic accession: one sequence was from ovaries at megasporogenesis stage; three were from megagametogensis stage; two were from both stages. Of the two sequences isolated from the sexual accessions, one showed expression in ovaries at megagametogensis, while the other sequence was shown to be specific to both stages.

Three sequences were from megasporogensis stage in apomicts but were also detected at megagametogensis in sexual plants.

Experimental systems for the molecular study of apomixis and sexuality have limitations mainly resulting from numerous differences in the genetic make up of the plant material. To overcome these limitations we examined mRNA populations extracted from ovaries pooled from different maturity stages. Hence the genes isolated from our study may not be specifically related to any stage of embryo sac development. The exact role of 1B8 can only be known after studying its in-situ expression level during megasporogenesis and megagametogenesis. The other genes implicated in our study showed homology to putative histone protein, receptor like protein kinase, cdc2-like protein kinase and a few unknown proteins. During northern analysis these clones have shown expression in both the apomictic and sexual ovaries. To analyze their copy number differences more sophisticated technique such as real time PCR could be employed, wherein the quantitative expression differences in the mRNA levels of apomictic and sexual ovaries can be estimated.

For the characterization of 11 selected clones, internal primers were designed. Successful amplification of all the 11cDNA fragments both in the recombinant plasmid (pBluescript KS+) and in the genome of *C. ciliaris* confirmed the presence of the respective genes.

5.8 Bioinformatic analysis

Database searches for selected 16 clones isolated from subtractive hybridization were carried out in order to localize homologue sequences and thereby infer putative protein functions. Little is known about the functions of proteins involved in apomictic development. The type of proteins showing homology to the genes isolated in our study are known to play pivotal role during cell cycle, cell division, reproduction and signal transduction mechanism. In previous studies where sequences were isolated by means of comparative gene expression between sexual and apomictic plants, relatively few clones were obtained and no clear function assigned (Chen *et al.*, 1999; Dusi, 2001; Pessino *et al.*, 2001). Some clues may be obtained from the functions of *fis* gene in *Arabidopsis*, which, in a way analogous to apomixis, lead to fertilization-independent seed development in the absence of fertilization (Chaudhury *et al.*, 1997). These genes code for Zn-finger containing transcriptional regulator and repressor proteins (Luo *et al.*,

1999). The genes for receptor like protein kinases are most closely related to MAP kinases, which represent proteins involved in the control of signal transduction pathways and gene expression, which are two basic processes important in cell differentiation. Hence, it is possible that the early onset of developmental programme can lead to a bypass of sexual reproduction, resulting in the expression of apomixis.

The relationships among different genes isolated in the present study were studied by dendrogram analysis. All the four-protein kinases cluster together, while bZIP protein was found to be close to pistil extensin protein and an unknown putative protein of rice. The cytoplasm male sterility factor as indicated by SCAR marker ApoF-08 has been found to be close to unknown protein and to putative histone H2A of rice. This indicated the possible similar functions associated with flower development, since it was close to pistil extensin protein.

The only clone, which showed expression in the apomictic ovary in the present study during reverse northern analysis, encodes a bZIP transcriptional factor, which belongs to a group of transcription factor defined by basic leucine zipper motif. The ZIP domains form two parallel-helices, arranged as coiled-coils and enabled dimerisation of two proteins (Baxevanis and Vinson, 1993), which determine the orientation of the basic domains within the major groove of the DNA. The functional role of bZIP transcription factors has been linked to a large diversity of biological aspects for example response to light (Oyama *et al.*, 1997; Schulze-Lefert *et al.*, 1989), hormones (Fukazawa *et al.*, 2000), biotic (Zhou *et al.*, 2000) and abiotic stresses (Aguan *et al.*, 1993) as well as cell proliferation (Mikami *et al.*, 1995) and developmental processes (Chuang *et al.*, 1999).

In order to characterize the secondary structure prediction and post- translational modification sites, various softwares were used. Many α-helices, β-turns and random coils were observed in the secondary structure. Izawa *et al.*, (1994) have shown the expression of bZIP transcriptional activator in developing rice seeds indicating its role in the regulation of genes during seed development. bZIP transcription factors have also been implicated in the flower development (Jakoby *et al.*, 2002). As in other TF families, many bZIP proteins probably have overlapping functions. Our identification of all bZIP genes is thus a necessary prerequisite for the dissection of individual bZIP function. Towards this we have analyzed various bZIP transcription factors using DNASTAR, which has indicated twenty-one other bZIP family genes. 1B8 clone showed maximum similarity with putative bZIP transcription factor of *Oryza sativa*. Whereas, it is very

distant from bZIP transcription factor RF2B of *Oryza sativa.* While analyzing the sequences of 75 putative AtbZIPs, Jakoby *et al.* (2002), defined ten groups of related proteins in which functional overlaps are most probable. The next step in understanding bZIP relationship will be to compare bZIP expression pattern, especially within a given group to detect potential overlapping functions. Data derived from monocot and dicot species suggested that homologues of group S-bZIPs are also transcriptionally activated after stress treatment (Kusano *et al.*, 1995) or specifically expressed in defined parts of the flower (Martinez *et al.*, 1998).

The conserved domain database is a compilation of multiple sequence alignments representing protein domains conserved in molecular evolution. Detailed analysis of the protein domains of the genes involved in the reproduction will help in deciphering the function of regulatory and structural genes. Towards this the conserved domains in the translated 1B8 clone was analyzed by domain finder, which indicated a conserved bZIP domain, which was 21 amino acid long. Another domain that was frequently observed among most of the genes isolated in this study pertained to receptor kinases. Understanding of the conserved domains will also help in secondary structure prediction and post translational modification sites.

SUMMARY

SUMMARY

Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform populations and perpetuates hybrid vigor through successive seed generations. The identification of the genes involved in apomixis appears to be tractable, because in most of the native apomictic systems under study, only a small number of loci have been determined to be critical for the inheritance of the trait. In the present study an attempt was made to isolate genes associated with apomixis from a naturally occurring obligate apomict *Cenchrus ciliaris.* A mapping population that segregated for apomixis and sexuality was developed by selfing three different facultative apomictic halfsibs. These halfsibs were generated from a sexually reproducing mother plant. Two approaches were followed *viz.* marker based DNA fingerprinting and protein and isozyme polymorphism studies. While eight sexual and five apomictic F2 segregants were used for DNA fingerprinting, two genotypes were used for subtractive hybridization, protein and isozyme analysis.

SDS-PAGE analysis of pre-meiotic and post-meiotic sexual and apomictic ovary extracts of F2 segregants indicated four differentially expressed proteins of molecular weights 97KDa, 80KDa, 60KDa and 50KDa, which were associated with different stages of megasporogenesis and megagametogenesis. Similarly, isozymes of Esterase and Phosphoglucomutase exhibited maximum polymorphism. EST-1 locus was associated with pre-meiotic stages, while three alleles of EST-1 were absent in the ovaries of sexual plant. Phylogenetic analysis using dendrogram showed different patterns of clustering for proteins and isozymes. The differentiation of proteins were more specific to pre-meiotic and post-meiotic stages of sexual and apomictic ovaries, whereas zymogram analysis clearly delineated pre-meiotic apomictic and sexual stages.

RAPD analysis of mapping population using six selected polymorphic primers indicated 36 DNA fingerprints that could discriminate apomictic and sexual F2 segregants. Out of these, one marker namely ApoF08 was found to be very closely associated with apomixis and hence was converted to a SCAR marker. Further analysis of SCAR marker by southern hybridization, indicated two loci for this marker, which

could be due to the segmental allopolyploidy nature of *C. ciliaris*. Overall DNA polymorphism was low due to more genetically homogenous F2 segregants. The population genetic parameters such as observed and effective number of alleles per locus, gene diversity and Shannon's information index and percent polymorphism showed higher amount among sexual segregants. Primer OPF-08 was the most polymorphic primer with a maximum polymorphism information content. The number of electrophoretic phenotypes were more among sexual segregants than apomictic segregants indicating higher amount of genetic variation. Again, dendrogram analysis indicated two clusters each comprising of apomictic and sexual segregants validating the discriminating power of six primers used in the present study.

Using subtractive hybridization, one cDNA clone of size 1029 bp showing homology to bZIP transcription factor was isolated which was further confirmed by reverse northern analysis. Many other genes, which were isolated by subtractive hybridization, did either show expression in both apomictic and sexual ovaries or showed no expression in apomictic and sexual ovaries. During earlier studies, bZIP transcription factors have been implicated in the flower development. Dendrogram analysis of different genes isolated in this study showed clustering of protein kinases, while bZIP protein was found to be close to the pistil extensin protein. 1B8 clone isolated in our study showed maximum similarity with putative bZIP transcription factor of *Oryza sativa*.

In order to determine the exact function of 1B8 clone, its *in situ* expression level during megasporogenesis and megagametogenesis has to be studied. For other promising clones isolated in the study, the quantitative expression differences between apomictic and sexual ovaries is required. Detailed protein analysis of 1B8 may help us to elucidate its putative function. Towards map based cloning more SCAR markers linked to apomixis may be developed by using larger mapping population. The proteins which were found associated with apomixis can be further characterized to determine their functions. Hence, the results of the present study are useful towards the isolation of genes controlling apomixis.

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APPENDICES

APPENDIX-1

COMPOSITION OF BUFFERS

20 X SSC (1000 ml)

NaCl	175.3 g
Sodium citrate (pH 7.0)	88.2 g

20 X SSPE (1000 ml)

NaCl	175.3 g
NaH_2PO_4	27.6 g
EDTA (pH 7.4)	7.4 g

5 X TBE buffer

Tris-base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0 ml

1M Sodium Phosphate Buffer (pH 7.2)

1M Na_2HPO_4	68.4 ml
1M NaH_2PO_4	31.6 ml

10X MOPS buffer (1000ml)

Morpholinopropanesulfonic acid	83.7gm
Sodium acetate, trihydrate	13.6gm
EDTA	3.72gm

50X Denhardts reagent (500ml)

Ficol (type 400)	5gm
Polyvinylpyrrolidone	5gm
Bovine serum albumin	5gm

Acrylamide/Bisacrylamide (30% stock)

Acrylamide	29gm
Bisacrylamide	1gm

6X Dye

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	0.25%

APPENDIX-2

Appendix 2A. BLAST analysis of 1B8 clone

BLASTX 2.2.9 [May-01-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1097744839-22421-212877976046.BLASTQ4

Query=
1b8ATGCTAGCATATAATGTACCTCTATTTAATCATTATGTAGCGACGATCATCACCATCATCATCATTA
GTGAAAACCTTGC
(651 letters)

Database: All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples
2,089,482 sequences; 702,241,455 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Sequences producing significant alignments:	Score (bits)	E Value
gi|53828635|gb|AAU94427.1| At1g58110 [Arabidopsis thaliana]	139	4e-32
gi|42562802|ref|NP_176108.3| bZIP family transcription fact	139	4e-32
gi|25404190|pir||D96614 hypothetical protein T18I24.3 [impo	130	3e-29
gi|18399693|ref|NP_564460.1| bZIP family transcription fact	91	2e-17
gi|8778353|gb|AAF79361.1| F15O4.49 [Arabidopsis thaliana] >	91	2e-17
gi|21592919|gb|AAM64869.1| unknown [Arabidopsis thaliana]	91	2e-17
gi|30984556|gb|AAP42741.1| At3g58120 [Arabidopsis thaliana]	77	4e-13
gi|30689007|ref|NP_850369.1| bZIP transcription factor fami	76	5e-13
gi|18491297|gb|AAL69473.1| At2g42380/MHK10.10 [Arabidopsis	76	5e-13
gi|34903896|ref|NP_913295.1| unnamed protein product [Oryza	74	2e-12
gi|50906641|ref|XP_464809.1| putative bZIP transcription fa	71	2e-11
gi|4567310|gb|AAD23721.1| expressed protein [Arabidopsis th	63	5e-09
gi|30689003|ref|NP_565970.2| bZIP transcription factor fami	63	5e-09
gi|29367343|gb|AAO72544.1| bZIP-like protein [Oryza sativa	61	2e-08
gi|50252666|dbj|BAD28835.1| bZIP protein-like [Oryza sativa	60	3e-08
gi|2921823|gb|AAC04862.1| shoot-forming PKSF1 [Paulownia ka	58	1e-07
gi|23297471|gb|AAN12977.1| unknown protein [Arabidopsis tha	57	3e-07
gi|19423874|gb|AAL87314.1| unknown protein [Arabidopsis tha	57	3e-07
gi|7270873|emb|CAB80553.1| putative protein [Arabidopsis th	57	3e-07
gi|30984520|gb|AAP42723.1| At5g04840 [Arabidopsis thaliana]	56	6e-07
gi|9758459|dbj|BAB08988.1| unnamed protein product [Arabido	56	6e-07

Appendix 2B. BLAST analysis of APOF-08 SCAR

BLASTN 2.2.8 [Jan-05-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1083422794-21537-141587249433.BLASTQ3

Query= Apof8
(591 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
2,198,014 sequences; 10,493,848,436 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Sequences producing significant alignments:	Score (bits)	E Value
gi|21210100|gb|AY107022.1| Zea mays PCO102665 mRNA sequence	46	0.080
gi|18542433|gb|AF468001.1| Mycoplasma hominis cell division	46	0.080
gi|45558547|gb|AY560328.1| Acinetobacter baumannii beta-lac	46	0.080
gi|42741401|gb|AY534236.1| Bacterium BPT-22 clone 22-3 inte	46	0.080
gi|42741400|gb|AY534235.1| Bacterium BPT-22 clone 22-1 inte	46	0.080
gi|21591827|gb|AC113619.5| Drosophila melanogaster X BAC RP	44	0.31
gi|21263184|gb|AC023710.5| Drosophila melanogaster X BAC RP	44	0.31
gi|407028|emb|Z26824.1|ZMFNRBP Zea mays gene for Ferredoxin	44	0.31
gi|17946652|gb|AY071735.1| Drosophila melanogaster RH45308	44	0.31
gi|21950715|gb|AY117410.1| Pennisetum glaucum isolate RGP	42	1.2
gi|18092333|gb|AF448416.1| Zea mays B73 chromosome 9S bz ge	42	1.2
gi|21239229|gb|AF387096.1| Meloidogyne incognita sequence c	42	1.2
gi|7899273|emb|AJ277413.1|BFR277413 Bacteroides fragilis in	42	1.2
gi|18568260|gb|AF466646.1| Zea mays clone ZMMBBb_Z195D10 pu	42	1.2
gi|21218103|dbj|AB074951.1| Uncultured delta proteobacteriu	42	1.2
gi|41393748|gb|AF215823.2| Zea mays T cytoplasm male steril	42	1.2
gi|37812559|gb|AY337922.1| Oryza sativa clone sk77 NBS-LRR-	42	1.2
gi|37812556|gb|AY337919.1| Oryza sativa clone sk103 NBS-LRR	42	1.2
gi|25988610|gb|AF454581.1| Leccinum rotundifoliae from Norw	40	4.9
gi|25988609|gb|AF454580.1| Leccinum vulpinum from Norway 18	40	4.9
gi|16225210|gb|AF416317.1|AF416317 Zea mays clone mPIF335 m	40	4.9
gi|45861074|gb|AC137967.14| Mus musculus chromosome 10, clo	40	4.9